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# Supplemental Data

# An Uncharged Amine in the Transition State of the Ribosomal Peptidyl Transfer Reaction

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## Supplemental Experimental Procedures and Results

This supplementary material includes kinetic and synthetic data. The kinetic experiments include: (i) representative 50S modified fragment assay results, (ii) primary data to demonstrate the independence of reaction rate on DMSO concentration within 70S ribosomes, and (iii) representative 70S initiation complex assay results. A complete synthetic description and chemical characterization for the various puromycin derivatives used in this study are also described.

### PRIMARY KINETIC DATA

*i. Representative 50S data*

Cytidine-puromycin (CPmn) derivatives were reacted with $^{32}$P 5'-end labeled CCA-pcb (P-site substrate) and 50S ribosomes at pH 8.5. The reaction substrates and products were separated under low-pH PAGE conditions to minimize background CCA-pcb hydrolysis (Figure S1A). The reaction rate ($k_{pep}$) was determined by plotting the fraction unreacted versus time (Figure S1B).

*ii. Effect of DMSO on 70S reaction rate*

Several of the derivatives were not sufficiently soluble in water to reach a saturating concentration in the 70S assay. DMSO was added to the reactions to increase reagent solubility. A DMSO concentration of 60% was found to be sufficient to solubilize the puromycin derivatives without significantly affecting the reaction rate. Puromycin (Pmn) was reacted with f[$^3$H]Met-tRNA$^{fMet}$ in 70S initiation complexes at pH 8.5 using a quench-flow apparatus in the presence of 0% and 60% DMSO. After quenching and extraction, dipeptide formation was quantitated by radioactive counting and the data fit as described in Materials and Methods (Figures S2A and S2B). 60% DMSO does not affect $k_{pep}$ for the 70S puromycin reaction (12 sec$^{-1}$ without DMSO, 13 sec$^{-1}$ with DMSO) and only slightly increases the apparent $K_M$ (2.5 mM without DMSO, 6.1 mM with DMSO) (Figures S2C and S2D).

*iii. Representative 70S data*

Puromycin (Pmn) derivatives were reacted with f[$^3$H]Met-tRNA$^{fMet}$ in the 70S initiation complex assay as described above.

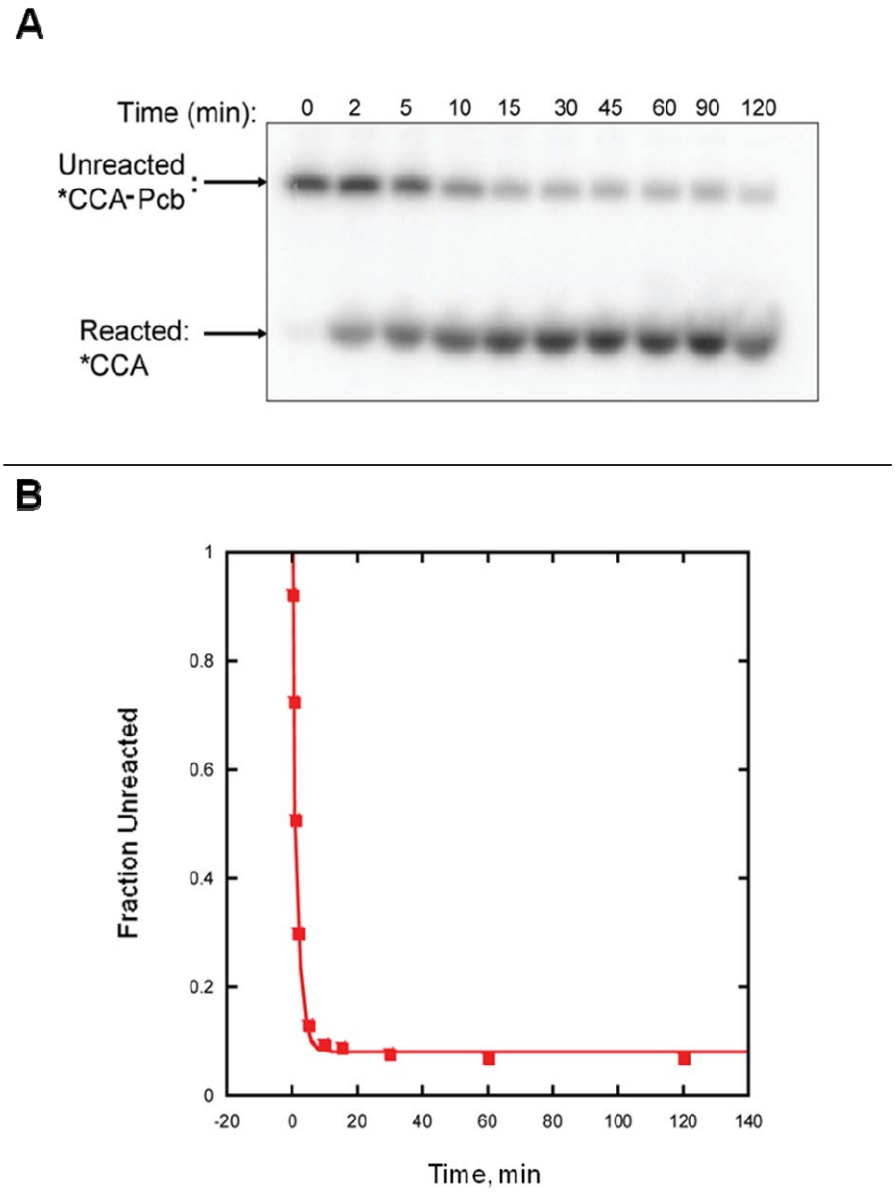



**Figure S1.** CPmn Derivative Rate Constant ($k_{pep}$) Determination with the 50S Modified Fragment Assay

(A) Representative polyacrylamide gel (12%) used to resolve unreacted *CCA-pcb from reacted *CCA. (B) Representative data obtained from PAGE phosphorimager quantification. $k_{pep}$ values are obtained by fitting the data to a single exponential.

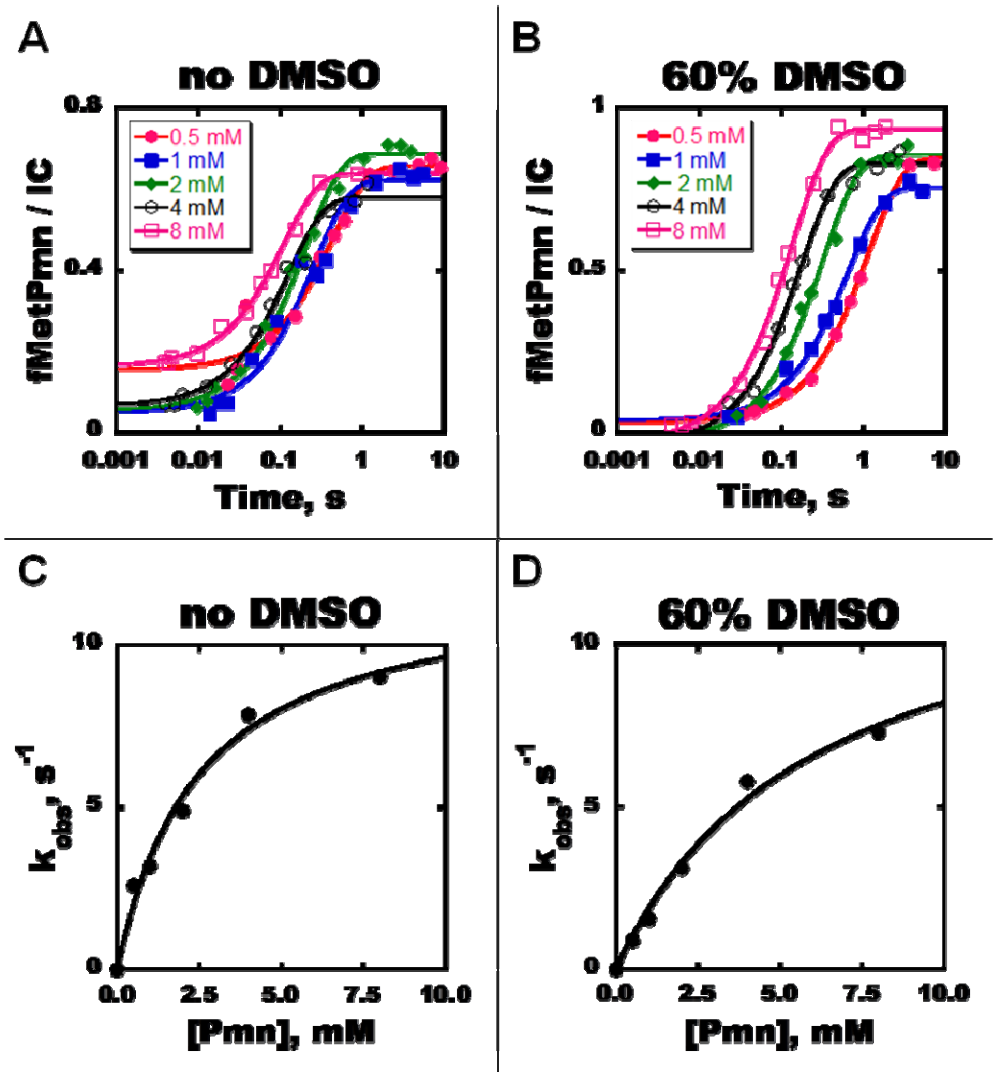



**Figure S2.** DMSO Effect on fMetPmn Formation for the 70S Initiation Complex (IC) Assay

Quench-flow assays were performed using 70S initiation complexes (0.1 μM) in buffer B (see Materials and Methods) at 37 ˚C and Pmn concentrations of 0.5 mM (●), 1 mM (■), 2 mM (♦), 4 mM (○), and 8 mM (□). (A) Time courses in the absence of DMSO. (B) Time courses in the presence of 60% DMSO. $k_{obs}$ values for individual curves were obtained by single-exponential fitting. (C) Concentration dependence of $k_{obs}$ in the absence of DMSO. (D) Concentration dependence of $k_{obs}$ in the presence of 60% DMSO. Fitting the data to a two-step model with a reversible binding step followed by an irreversible reaction yields $k_{pep}$ and $K_M$ values. IC, 70S initiation complex.

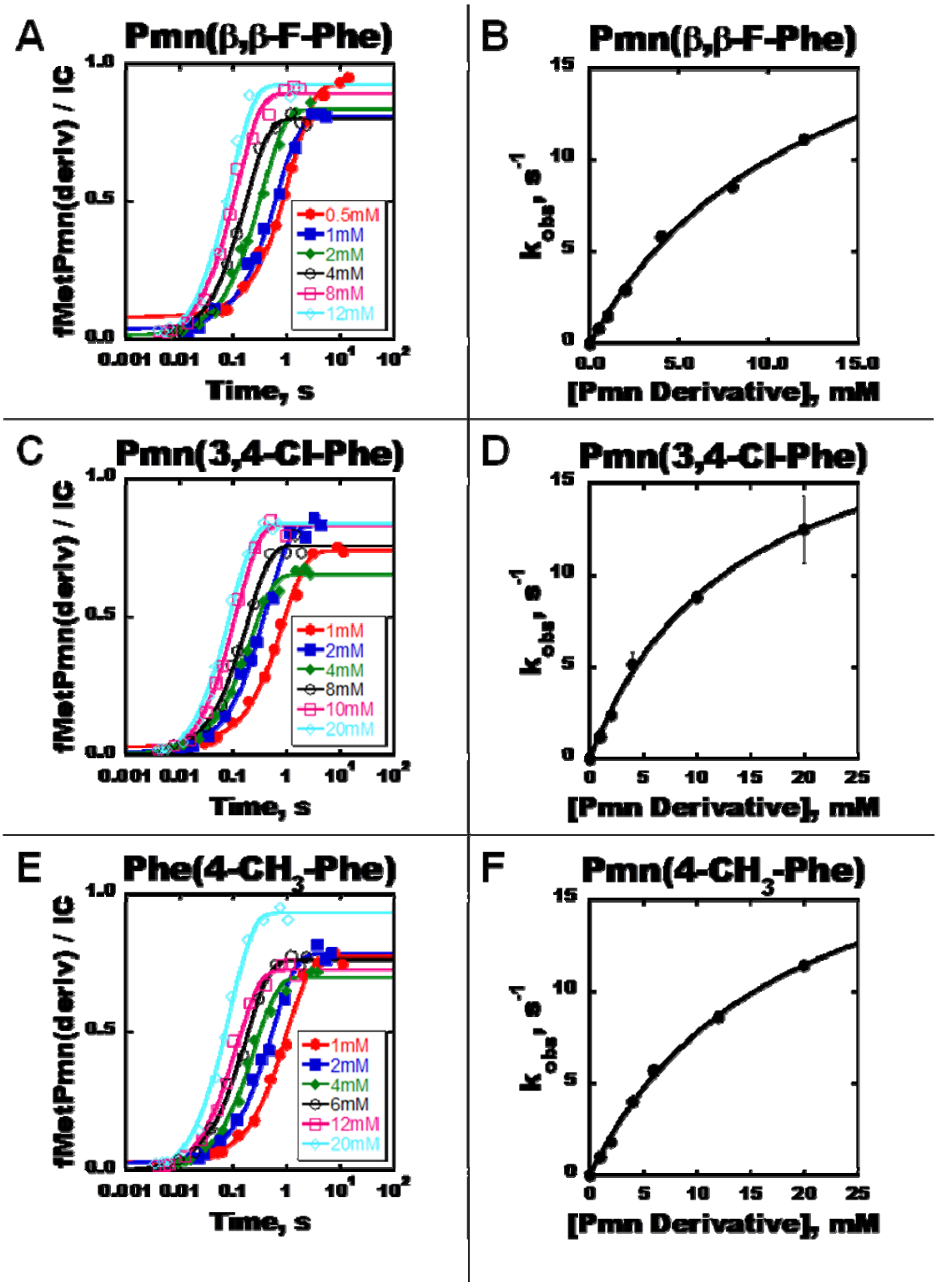
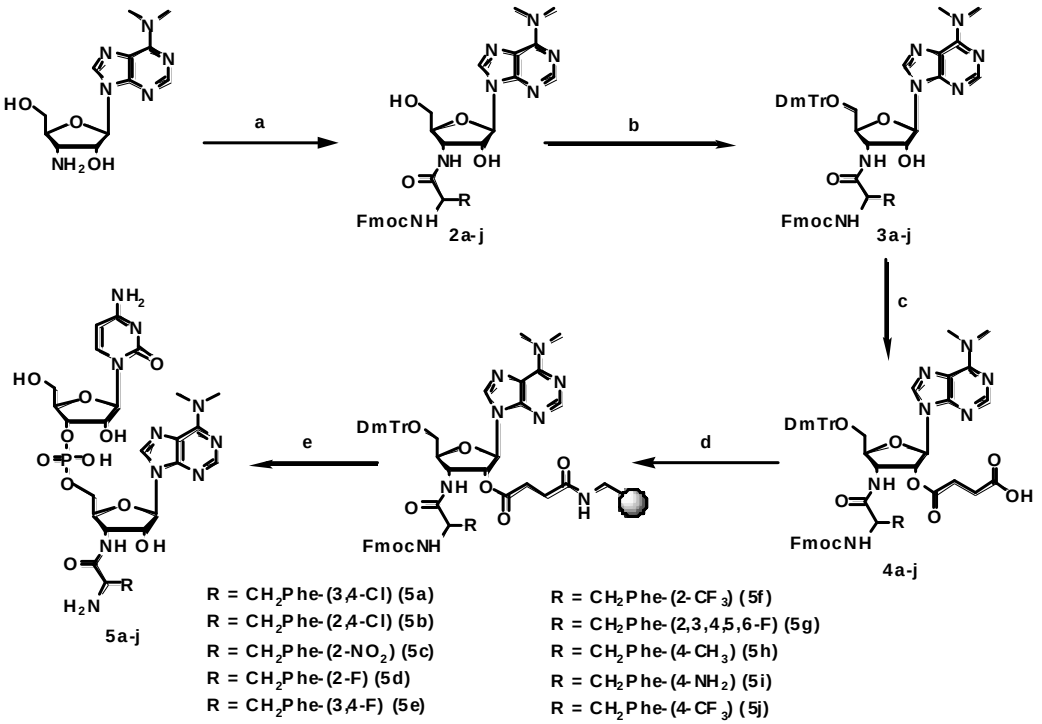



**Figure S3.** Rate Constant of Reaction ($k_{pep}$) and Apparent Affinity to the Ribosome ($K_M$) of Pmn Derivatives in the 70S Initiation Complex Assay
Representative time courses (A, C, E) and $k_{obs}$ concentration dependencies (B, D, F) for: (A)&(B) Puromycin(β,β-F-Phe); (C)&(D) Puromycin(3,4-Cl-Phe); and (E)&(F) Puromycin(4-$CH_3$-Phe). All data were fit as above.

**SYNTHETIC SCHEME**

Puromycin (Pmn) derivatives were prepared by solution phase chemistry (Scheme 1 a) and Cytidine-puromycin (CPmn) derivatives were prepared by combining solution phase synthesis and solid phase synthesis (scheme 1 a-e). After activation of N-Fmoc protected amino-acids by cyanuric fluoride, the corresponding acyl fluorides **1a-g** were coupled to puromycin aminonucleoside to generate the puromycin derivatives **2a-g** in good yield (method 1, 87 – 99%). Another coupling method using N-hydroxysuccinimide (SuOH) and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI) was also used. In this case, nucleosides analogs **2h-j** were obtained in moderate yield (method 2, 85 – 87%). For Pmn derivatives, nucleoside analogs **2a-j** were deprotected to produce the final compound . For CPmn derivatives, nucleoside analogs **2a-j** were protected at the 5'-position by the acid-labile DmTr protecting group (compounds **3a-j**) and then attached to the succinate linker at the 2'-position. The resulting succinate derivatives **4a-j** were derivatized to the polymer support and used in solid phase synthesis. The protected CPmn derivatives were cleaved from the support, deprotected and purified by reverse HPLC to produce pure CPmn analogues **5a-j**.



Scheme 1: (a) method 1: acyl fluoride **1a-g**, cyanuric fluoride, Pyridine, DMF, RT, 3h, 87 – 99%. method 2: N-Fmoc-amino-acid, SuOH, EDCI, DMF, RT, 24h, 85 – 87%. (b) DmTrCl, Pyridine, $NEt_3$, RT, 3h, 40 – 79%. (c) succinic anhydride, DMAP, Pyridine, RT, overnight, 62 – 85%. (d) Amino derivatized Primer Support, EDCI, DMAP, Pyridine, $NEt_3$, RT. (d) solid phase synthesis.

**METHODS**

All reactions were monitored by thin-layer chromatography (TLC) using E. Merck silica gel 60 F254 precoated plates (0.25 mm). Flash chromatography was performed with the indicated solvent system using Silicycle 0.040-0.060 mm silica gel. NMR spectra were measured on Brucker Avance DPX-400 and Brucker Avance DPX-500 spectrometers. $^{1}H$ and $^{13}C$ NMR chemical shifts were recorded using tetramethylsilane as an internal standard and $^{19}F$ NMR chemical shifts were recorded using trichlorofluoromethane as an internal standard. Mass spectra were collected on Waters Micromass LCT and Waters Micromass ZQ mass spectrometers. Chemicals and anhydrous solvents were used as received from commercial suppliers. Protected amino-acids and puromycin aminonucleoside were purchased from Sigma-Aldrich or CSPS pharmaceuticals.

**General Procedure for the Preparation of Acyl Fluoride Derivatives 1a-g**

In a 50 ml round bottom flask containing the N-(9-fluorenylmethoxycarbonyl)-amino acid (1 eq), anhydrous dichloromethane, anhydrous pyridine (1.5 eq), and cyanuric fluoride (3 eq) were added under argon. The solution was stirred 24 h at room temperature, then diluted into dichloromethane (60 ml) and extracted with cold water (3 x 25 ml). The organic layer was dried with anhydrous $MgSO_4$, filtered and concentrated. The residue was recrystallized from dichloromethane/hexane to give the corresponding acyl fluoride derivatives **1a-g**.

*N-(9-Fluorenylmethoxycarbonyl)-L-3,4-dichlorophenylalanyl Fluoride* **1a**. Isolated in 79% yield (395 mg), starting from N-(9-Fluoroenylmethoxycarbonyl)-3,4-dichlorophenylalanine (500 mg, 1.095 mmol), anhydrous dichloromethane (14 ml), anhydrous pyridine (133 µl, 1.643 mmol), cyanuric fluoride (445 mg, 3.285 mmol): $^{1}H$

NMR (400 MHz, DMSO-d6): 8.19 (d, 1H, $^{3}J_{HH}$ = 7.6 Hz), 7.93 (d, 2H, $^{3}J_{HH}$ = 7.5 Hz), 7.63 (m, 3H), 7.58 (d, 1H, $^{3}J_{HH}$ = 8.2 Hz), 7.45 (t, 2H, $^{3}J_{HH}$ = 7.4 Hz), 7.34 (q, 2H, $^{3}J_{HH}$ = 6.4 Hz), 7.25 (dd, 1H, $^{4}J_{HH}$ = 1.9 Hz, $^{3}J_{HH}$ = 8.2 Hz), 4.69 (m, 1H), 4.43 (dd, 1H, $^{3}J_{HH}$ = 6.7 Hz, $^{2}J_{HH}$ = 10.6 Hz), 4.34 (dd, 1H, $^{3}J_{HH}$ = 6.7 Hz, $^{2}J_{HH}$ = 10.5 Hz), 4.24 (t, 1H, $^{3}J_{HH}$ = 6.5 Hz), 3.21 (dd, 1H, $^{3}J_{HH}$ = 4.6 Hz, $^{2}J_{HH}$ = 13.8 Hz), 3.02 (dd, 1H, $^{3}J_{HH}$ = 10.6 Hz, $^{2}J_{HH}$ = 13.7 Hz). $^{19}$F NMR (376 MHz, DMSO-d6): 29.45 (s, 1F). $^{13}$C NMR (125 MHz, DMSO-d6): 164.12 (d, $^{1}J_{CF}$ = 372.6 Hz), 158.03, 145.74, 145.72, 142.92, 142.90, 139.94, 133.48, 132.97, 132.53, 131.92, 131.68, 129.81, 129.21, 129.17, 127.20, 127.13, 122.31, 67.96, 55.62 (d, $^{2}J_{CF}$ = 53.1 Hz), 48.73, 36.21

*N-(9-Fluorenylmethoxycarbonyl)-L-2,4-dichlorophenylalanyl Fluoride* **1b**. Isolated in 88% yield (445 mg), starting from N-(9-Fluoroenylmethoxycarbonyl)-2,4-dichlorophenylalanine (500 mg, 1.095 mmol), anhydrous dichloromethane (14 ml), anhydrous pyridine (133 µl, 1.643 mmol), cyanuric fluoride (445 mg, 3.285 mmol): $^{1}$H NMR (400 MHz, DMSO-d6): 7.98 (d, 1H, $^{3}J_{HH}$ = 7.6 Hz), 7.68 (d, 2H, $^{3}J_{HH}$ = 7.5 Hz), 7.41 (m, 3H), 7.21 (t, 3H, $^{3}J_{HH}$ = 7.4 Hz), 7.12 (m, 3H), 4.38 (m, 1H), 4.21 (dd, 1H, $^{3}J_{HH}$ = 6.6 Hz, $^{2}J_{HH}$ = 10.5 Hz), 4.11 (dd, 1H, $^{3}J_{HH}$ = 6.7 Hz, $^{2}J_{HH}$ = 10.5 Hz), 3.99 (t, 1H, $^{3}J_{HH}$ = 6.5 Hz), 3.06 (dd, 1H, $^{3}J_{HH}$ = 5.0 Hz, $^{2}J_{HH}$ = 14.0 Hz), 2.94 (dd, 1H, $^{3}J_{HH}$ = 10.3 Hz, $^{2}J_{HH}$ = 13.9 Hz). $^{19}$F NMR (376 MHz, DMSO-d6): 29.15 (s, 1F). $^{13}$C NMR (100 MHz, DMSO-d6): 162.17 (d, $^{1}J_{CF}$ = 372.2 Hz), 156.24, 143.96, 143.92, 141.13, 141.11, 134.71, 133.51, 133.48, 132.92, 129.14, 128.02, 127.72, 127.42, 127.39, 125.43, 125.36, 120.52, 66.18, 52.48 (d, $^{2}J_{CF}$ = 53.3 Hz), 46.91, 32.83.

*N-(9-Fluorenylmethoxycarbonyl)-L-2-nitrophenylalanyl fluoride* **1c**. Isolated in 95% yield (480 mg), starting from N-(9-Fluoroenylmethoxycarbonyl)-L-2-nitrophenylalanine (500 mg, 1.156 mmol), anhydrous dichloromethane (15 ml), anhydrous pyridine (140 µl, 1.734 mmol), cyanuric fluoride (470 mg, 3.468 mmol): $^{1}$H NMR (400 MHz, $CDCl_3$): 8.02 (d, 1H, $^{3}J_{HH}$ = 8.1 Hz), 7.75 (d, 2H, $^{3}J_{HH}$ = 7.5 Hz), 7.25 – 7.56 (m, 10H), 5.53 (d, 1H, $^{3}J_{HH}$ = 8.1 Hz), 4.48 (m, 1H), 4.35 (m, 1H), 4.15 (t, 1H, $^{3}J_{HH}$ = 6.7 Hz), 3.61 (dd, 1H, $^{3}J_{HH}$ = 5.3 Hz, $^{2}J_{HH}$ = 13.7 Hz), 3.37 (dd, 1H, $^{3}J_{HH}$ = 9.5 Hz, $^{2}J_{HH}$ = 13.6 Hz). $^{19}$F NMR (376 MHz, $CDCl_3$): 30.91 (s, 1F). $^{13}$C NMR (100 MHz, $CDCl_3$): 161.37 (d, $^{1}J_{CF}$ = 370.4 Hz),

155.57, 149.43, 143.49, 143.44, 141.30, 133.67, 132.91, 130.40, 128.94, 127.82, 127.10, 125.45, 125.00, 124.94, 120.03, 67.42, 53.56 (d, $^{2}J_{CF}$ = 58.8 Hz), 46.95, 34.16.

*N-(9-Fluorenylmethoxycarbonyl)-L-2-fluorophenylalanyl fluoride* **1d**. Isolated in 95% yield (480 mg), starting from N-(9-Fluoroenylmethoxycarbonyl)-L-2-fluorophenylalanine (500 mg, 1.233 mmol), anhydrous dichloromethane (16 ml), anhydrous pyridine (150 µl, 1.849 mmol), cyanuric fluoride (500 mg, 3.699 mmol): $^{1}$H NMR (400 MHz, DMSO-d6): 8.24 (d, 1H, $^{3}J_{HH}$ = 7.5 Hz), 7.90 (d, 2H, $^{3}J_{HH}$ = 7.5 Hz), 7.64 (t, 2H, $^{3}J_{HH}$ = 6.7 Hz), 7.43 (t, 2H, $^{3}J_{HH}$ = 7.4 Hz), 7.33 (m, 4H), 7.15 (m, 2H), 4.57 (m, 1H), 4.40 (dd, 1H, $^{3}J_{HH}$ = 6.9 Hz, $^{2}J_{HH}$ = 10.4 Hz), 4.31 (dd, 1H, $^{3}J_{HH}$ = 6.7 Hz, $^{2}J_{HH}$ = 10.4 Hz), 4.21 (t, 1H, $^{3}J_{HH}$ = 6.6 Hz), 3.21 (dd, 1H, $^{3}J_{HH}$ = 5.4 Hz, $^{2}J_{HH}$ = 14.0 Hz), 3.11 (dd, 1H, $^{3}J_{HH}$ = 9.9 Hz, $^{2}J_{HH}$ = 13.7 Hz). $^{19}$F NMR (376 MHz, DMSO-d6): 29.79 (s, 1F), -118.07 (s, 1F). $^{13}$C NMR (100 MHz, $CDCl_3$): 161.76 (d, $^{1}J_{CF}$ = 344.0 Hz), 161.14 (d, $^{1}J_{CF}$ = 272.0 Hz), 143.54, 141.29, 131.52 (d, $^{3}J_{CF}$ = 4.0 Hz), 129.77 (d, $^{3}J_{CF}$ = 8.0 Hz), 127.79, 127.09, 125.00, 124.67 (d, $^{4}J_{CF}$ = 4.0 Hz), 121.60 (d, $^{2}J_{CF}$ = 16.0 Hz), 120.03, 115.73 (d, $^{2}J_{CF}$ = 22.0 Hz), 67.40, 53.13 (d, $^{2}J_{CF}$ = 60.0 Hz), 47.00, 30.54.

*N-(9-Fluorenylmethoxycarbonyl)-L-3,4-difluorophenylalanyl fluoride* **1e**. Isolated in 97% yield (490 mg), starting from N-(9-Fluoroenylmethoxycarbonyl)-L-3,4-difluorophenylalanine (500 mg, 1.180 mmol), anhydrous dichloromethane (15 ml), anhydrous pyridine (150 µl, 1.770 mmol), cyanuric fluoride (480 mg, 3.540 mmol): $^{1}$H NMR (400 MHz, DMSO-d6): 8.16 (d, 1H, $^{3}J_{HH}$ = 7.5 Hz), 7.90 (d, 2H, $^{3}J_{HH}$ = 7.5 Hz), 7.62 (d, 2H, $^{3}J_{HH}$ = 7.3 Hz), 7.29 – 7.44 (m, 6H), 7.08 (m, 1H), 4.63 (m, 1H), 4.42 (dd, 1H, $^{3}J_{HH}$ = 6.8 Hz, $^{2}J_{HH}$ = 10.6 Hz), 4.33 (dd, 1H, $^{3}J_{HH}$ = 6.6 Hz, $^{2}J_{HH}$ = 10.5 Hz), 4.21 (t, 1H, $^{3}J_{HH}$ = 6.5 Hz), 3.17 (dd, 1H, $^{3}J_{HH}$ = 4.8 Hz, $^{2}J_{HH}$ = 13.9 Hz), 2.99 (dd, 1H, $^{3}J_{HH}$ = 10.5 Hz, $^{2}J_{HH}$ = 13.8 Hz). $^{19}$F NMR (376 MHz, DMSO-d6): 30.08 (s, 1F), -138.98 (d, 1F, $^{3}J_{FF}$ = 22.4 Hz), -141.50 (d, 1F, $^{3}J_{FF}$ = 22.3 Hz). $^{13}$C NMR (100 MHz, DMSO-d6): 162.02 (d, $^{1}J_{CF}$ = 372.8 Hz), 155.86, 149.05 (dd, $^{2}J_{CF}$ = 12.5 Hz, $^{1}J_{CF}$ = 244.9 Hz), 148.44 (dd, $^{2}J_{CF}$ = 12.5 Hz, $^{1}J_{CF}$ = 245.1 Hz), 143.55, 140.75, 140.73, 134.28 (dd, $^{4}J_{CF}$ = 4.0 Hz, $^{3}J_{CF}$ = 5.7 Hz), 127.64, 127.01, 126.97, 126.18 (dd, $^{4}J_{CF}$ = 3.2 Hz, $^{3}J_{CF}$ = 6.3 Hz), 125.03, 124.96, 120.14, 118.19 (d, $^{2}J_{CF}$ = 17.0 Hz), 117.18 (d, $^{2}J_{CF}$ = 16.9 Hz), 65.72, 53.66 (d, $^{2}J_{CF}$ = 52.9 Hz), 46.54, 34.11.

*N-(9-Fluorenylmethoxycarbonyl)-L-2-trifluoromethylphenylalanyl fluoride* **1f**. Isolated in 95% yield (480 mg), starting from N-(9-Fluoroenylmethoxycarbonyl)-L-2-trifluoromethylphenylalanine (500 mg, 1.097 mmol), anhydrous dichloromethane (15 ml), anhydrous pyridine (133 µl, 1.645 mmol), cyanuric fluoride (445 mg, 3.291 mmol): $^{1}$H NMR (400 MHz, DMSO-d6): 8.29 (d, 1H, $^{3}J_{HH}$ = 7.7 Hz), 7.91 (d, 2H, $^{3}J_{HH}$ = 7.5 Hz), 7.73 (d, 1H, $^{3}J_{HH}$ = 7.7 Hz), 7.65 (t, 2H, $^{3}J_{HH}$ = 6.6 Hz), 7.60 (t, 1H, $^{3}J_{HH}$ = 7.3 Hz), 7.49 (m, 2H), 7.43 (t, 2H, $^{3}J_{HH}$ = 7.4 Hz), 7.33 (q, 2H, $^{3}J_{HH}$ = 6.9 Hz), 4.59 (m, 1H), 4.41 (dd, 1H, $^{3}J_{HH}$ = 6.7 Hz, $^{2}J_{HH}$ = 10.5 Hz), 4.33 (dd, 1H, $^{3}J_{HH}$ = 6.6 Hz, $^{2}J_{HH}$ = 10.5 Hz), 4.21 (t, 1H, $^{3}J_{HH}$ = 6.6 Hz), 3.35 (m, 1H), 3.23 (dd, 1H, $^{3}J_{HH}$ = 10.1 Hz, $^{2}J_{HH}$ = 14.1 Hz). $^{19}$F NMR (376 MHz, DMSO-d6): 29.45 (s, 1F), -57.97 (s, 3F). $^{13}$C NMR (100 MHz, DMSO-d6): 161.89 (d, $^{1}J_{CF}$ = 372.2 Hz), 155.92, 143.61, 140.80, 140.79, 134.71, 132.45, 132.26, 127.71, 127.66, 127.55 (q, $^{2}J_{CF}$ = 32.1 Hz), 127.11, 127.07, 125.97 (q, $^{3}J_{CF}$ = 5.6 Hz), 125.10, 125.05, 124.46 (q, $^{1}J_{CF}$ = 273.9 Hz), 120.20, 65.90, 53.53 (d, $^{2}J_{CF}$ = 53.6 Hz), 46.56, 31.88.

*N-(9-Fluorenylmethoxycarbonyl)-L-pentafluorophenylalanyl fluoride* **1g**. Isolated in 30% yield (150 mg), starting from N-(9-Fluoroenylmethoxycarbonyl)-L-pentafluorophenylalanine (500 mg, 1.047 mmol), anhydrous dichloromethane (13.5 ml), anhydrous pyridine (130 µl, 1.570 mmol), cyanuric fluoride (425 mg, 3.141 mmol): $^{1}$H NMR (400 MHz, DMSO-d6): 8.22 (d, 1H, $^{3}J_{HH}$ = 7.6 Hz), 7.90 (d, 2H, $^{3}J_{HH}$ = 7.5 Hz), 7.64 (t, 2H, $^{3}J_{HH}$ = 7.1 Hz), 7.43 (t, 2H, $^{3}J_{HH}$ = 7.4 Hz), 7.32 (t, 2H, $^{3}J_{HH}$ = 7.4 Hz), 4.66 (m, 1H), 4.37 (m, 2H), 4.21 (t, 1H, $^{3}J_{HH}$ = 6.6 Hz), 3.25 (m, 2H). $^{19}$F NMR (376 MHz, DMSO-d6): 28.41 (s, 1F), -141.87 (dd, 2F, $^{4}J_{FF}$ = 7.6 Hz, $^{3}J_{FF}$ = 23.6 Hz), -156.58 (t, 1F, $^{3}J_{FF}$ = 22.3 Hz), -163.15 (dt, 2F, $^{4}J_{FF}$ = 7.5 Hz, $^{3}J_{FF}$ = 23.4 Hz). $^{13}$C NMR (100 MHz, DMSO-d6): 161.07 (d, $^{1}J_{CF}$ = 369.0 Hz), 155.80, 146.40 (m), 143.94 (m), 143.56, 143.43, 140.71, 138.08 (m), 135.47 (m), 127.65, 126.98, 124.92, 120.12, 110.55 (m), 66.08, 51.31 (d, $^{2}J_{CF}$ = 52.4 Hz), 46.43, 23.21.

**General Procedure for the Preparation of Puromycin Derivatives 2a-g (Method 1)**
After co-evaporationing with pyridine (3 x 10ml), puromycin aminonucleoside (1 eq) was placed in a 50 ml round bottom flask under argon and dissolved with anhydrous DMF

and anhydrous pyridine. Acyl fluoride derivative **1a-g** (1.01 eq) was introduced and the solution was stirred at room temperature for 3 hours. After evaporation in vacuo, the oily residue was purified by flash chromatography to produce puromycin derivatives **2a-g**.

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-3,4-dichlorophenylalanyl]-$N^6$,$N^6$-dimethyladenosine* **2a**. Isolated in 94% yield (468 mg) by flash chromatography ($CH_2Cl_2$ /MeOH, 95:5), starting from puromycin aminonucleoside (200 mg, 0.680 mmol), acyl fluoride **1a** (342 mg, 0.686 mmol), anhydrous pyridine (1 ml), anhydrous DMF (19 ml). $^1$H NMR (500 MHz, DMSO-d6): 8.50 (s, 1H), 8.33 (d, 1H, $^3J_{HH}$ = 6.9 Hz), 8.29 (s, 1H), 7.92 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.72 (m, 2H), 7.66 (t, 2H, $^3J_{HH}$ = 7.1 Hz), 7.57 (d, 1H, $^3J_{HH}$ = 8.2 Hz), 7.39 – 7.46 (m, 3H), 7.33 (m, 2H), 6.23 (d, 1H, $^3J_{HH}$ = 4.2 Hz), 6.06 (d, 1H, $^3J_{HH}$ = 1.8 Hz), 5.24 (t, 1H, $^3J_{HH}$ = 5.3 Hz), 4.57 (m, 2H), 4.45 (m, 1H), 4.20 (m, 3H), 4.03 (m, 1H), 3.74 (m, 1H), 3.56 (m, 7H), 3.06 (dd, 1H, $^3J_{HH}$ = 3.6 Hz, $^2J_{HH}$ = 13.4 Hz), 2.84 (dd, 1H, $^3J_{HH}$ = 11.2 Hz, $^2J_{HH}$ = 12.0 Hz). $^{13}$C NMR (125 MHz, DMSO-d6): 171.89, 156.12, 154.64, 152.22, 150.03, 144.14, 144.01, 141.02, 140.99, 139.83, 138.26, 131.69, 130.88, 130.45, 130.22, 129.32, 127.96, 127.36, 125.63, 125.55, 120.45, 119.99, 89.79, 83.83, 73.40, 66.06, 61.29, 56.04, 55.26, 50.73, 46.87, 37.37. ESI-MS ($ES^+$): m/z calcd for $C_{36}H_{35}Cl_2N_7O_6$ 731.2, found 732.6 ($MH^+$), 754.5 ($M + Na^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-2,4-dichlorophenylalanyl]-$N^6$,$N^6$-dimethyladenosine* **2b**. Isolated in 87% yield (432 mg) by flash chromatography ($CH_2Cl_2$ /MeOH, 95:5), starting from puromycin aminonucleoside (200 mg, 0.680 mmol), acyl fluoride **1b** (342 mg, 0.686 mmol), anhydrous pyridine (1 ml), anhydrous DMF (19 ml). $^1$H NMR (500 MHz, DMSO-d6): 8.37 (s, 1H), 8.16 (s, 1H), 7.97 (d, 1H, $^3J_{HH}$ = 7.6 Hz), 7.81 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.62 (d, 1H, $^3J_{HH}$ = 8.9 Hz), 7.58 (d, 2H, $^3J_{HH}$ = 7.3 Hz), 7.49 (d, 1H, $^3J_{HH}$ = 2.1 Hz), 7.21 – 7.35 (m, 6H), 5.97 (d, 1H, $^3J_{HH}$ = 4.9 Hz), 5.92 (d, 1H, $^3J_{HH}$ = 3.1 Hz), 5.13 (t, 1H, $^3J_{HH}$ = 5.3 Hz), 4.38 – 4.47 (m, 3H), 4.05 – 4.15 (m, 3H), 3.88 (m, 1H), 3.38 – 3.62 (m, 8H), 3.09 (dd, 1H, $^3J_{HH}$ = 5.2 Hz, $^2J_{HH}$ = 14.0 Hz), 2.88 (dd, 1H, $^3J_{HH}$ = 9.8 Hz, $^2J_{HH}$ = 14.0 Hz). $^{13}$C NMR (125 MHz, $CDCl_3$): 171.36, 156.04, 154.64, 152.21, 150.05, 144.11, 144.04, 141.03, 141.02, 138.26, 134.78, 133.35, 133.09, 132.27, 129.27, 128.90, 127.99, 127.64, 127.39, 125.63, 125.59, 120.46,

119.98, 89.58, 83.63, 73.35, 66.17, 61.27, 55.27, 54.30, 50.78, 46.87, 35.34. ESI-MS ($ES^+$): m/z calcd for $C_{36}H_{35}Cl_2N_7O_6$ 731.2, found 732.6 ($MH^+$), 754.6 ($M + Na^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-2-nitrophenylalanyl]-$N^6$,$N^6$-dimethyladenosine* **2c**. Isolated in 99% yield (480 mg) by flash chromatography ($CH_2Cl_2$ /MeOH, 95:5), starting from puromycin aminonucleoside (200 mg, 0.680 mmol), acyl fluoride **1c** (298 mg, 0.686 mmol), anhydrous pyridine (1 ml), anhydrous DMF (19 ml). $^1$H NMR (500 MHz, DMSO-d6): 8.44 (s, 1H), 8.24 (s, 1H), 8.02 (d, 1H, $^3J_{HH}$ = 7.7 Hz), 7.98 (d, 1H, $^3J_{HH}$ = 8.0 Hz), 7.88 (d, 2H, $^3J_{HH}$ = 7.4 Hz), 7.71 (d, 1H, $^3J_{HH}$ = 8.8 Hz), 7.30 – 7.66 (m, 9H), 6.03 (d, 1H, $^3J_{HH}$ = 4.9 Hz), 5.98 (d, 1H, $^3J_{HH}$ = 3.1 Hz), 5.18 (t, 1H, $^3J_{HH}$ = 5.4 Hz), 4.45 – 4.59 (m, 3H), 4.11 – 4.22 (m, 3H), 3.92 (m, 1H), 3.44 – 3.68 (m, 8H), 3.37 (dd, 1H, $^3J_{HH}$ = 5.3 Hz, $^2J_{HH}$ = 14.1 Hz), 3.15 (dd, 1H, $^3J_{HH}$ = 9.6 Hz, $^2J_{HH}$ = 13.9 Hz). $^{13}$C NMR (125 MHz, DMSO-d6): 171.40, 156.07, 154.67, 152.22, 150.08, 149.69, 144.08, 141.05, 141.02, 138.28, 133.42, 132.86, 132.68, 129.28, 128.38, 128.00, 127.42, 125.66, 125.69, 124.94, 120.45, 120.02, 89.59, 83.73, 73.39, 66.20, 61.34, 54.92, 50.80, 46.90, 34.91. ESI-MS ($ES^+$): m/z calcd for $C_{36}H_{36}N_8O_8$ 708.2, found 709.2 ($MH^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-2-fluorophenylalanyl]-$N^6$,$N^6$-dimethyladenosine* **2d**. Isolated in 97% yield (450 mg) by flash chromatography ($CH_2Cl_2$ /MeOH, 95:5), starting from puromycin aminonucleoside (200 mg, 0.680 mmol), acyl fluoride **1d** (280 mg, 0.686 mmol), anhydrous pyridine (1 ml), anhydrous DMF (19 ml). $^1$H NMR (500 MHz, DMSO-d6): 8.43 (s, 1H), 8.22 (s, 1H), 8.06 (d, 1H, $^3J_{HH}$ = 7.7 Hz), 7.86 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.63 (m, 2H), 7.39 (t, 1H, $^3J_{HH}$ = 7.4 Hz), 7.23 – 7.35 (m, 6H), 7.11 (t, 1H, $^3J_{HH}$ = 9.5 Hz), 7.06 (t, 1H, $^3J_{HH}$ = 7.4 Hz), 6.03 (sbr, 1H), 5.97 (d, 1H, $^3J_{HH}$ = 3.1 Hz), 5.16 (t, 1H, $^3J_{HH}$ = 5.3 Hz), 4.42 – 4.51 (m, 3H), 4.10 – 4.22 (m, 3H), 3.92 (m, 1H), 3.43 – 3.67 (m, 8H), 3.08 (dd, 1H, $^3J_{HH}$ = 5.4 Hz, $^2J_{HH}$ = 14.0 Hz), 2.88 (dd, 1H, $^3J_{HH}$ = 9.7 Hz, $^2J_{HH}$ = 14.0 Hz). $^{19}$F NMR (376 MHz, DMSO-d6): -117.62 (s, 1F). $^{13}$C NMR (125 MHz, DMSO-d6): 171.60, 162.65, 161.13 (d, $^1J_{CF}$ = 244.2 Hz), 156.02, 154.65, 152.20, 150.06, 144.10, 144.09, 141.02, 141.00, 138.25, 131.96 (d, $^3J_{CF}$ = 4.3 Hz), 129.27, 128.83 (d, $^3J_{CF}$ = 8.0 Hz), 127.98, 127.63, 127.42, 127.40, 125.70, 125.59, 124.80 (d, $^2J_{CF}$ = 15.7 Hz), 124.39 (sbr), 121.73, 120.43, 120.37, 120.00, 115.33 (d, $^2J_{CF}$

= 21.9 Hz), 89.59, 83.68, 73.39, 66.16, 61.29, 55.26, 54.98, 50.76, 46.89, 36.12, 31.46, 31.12. ESI-MS ($ES^+$): m/z calcd for $C_{36}H_{36}FN_7O_6$ 681.2, found 682.6 ($MH^+$), 704.5 (M + $Na^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-3,4-difluorophenylalanyl]-$N^6$,$N^6$-dimethyladenosine* **2e**. Isolated in 98% yield (470 mg) by flash chromatography ($CH_2Cl_2$ /MeOH, 95:5), starting from puromycin aminonucleoside (200 mg, 0.680 mmol), acyl fluoride **1e** (292 mg, 0.686 mmol), anhydrous pyridine (1 ml), anhydrous DMF (19 ml). $^1$H NMR (400 MHz, DMSO-d6): 8.47 (s, 1H), 8.27 (d, 1H, $^3J_{HH}$ = 7.0 Hz), 8.25 (s, 1H), 7.88 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.69 (d, 1H, $^3J_{HH}$ = 8.8 Hz), 7.63 (d, 2H, $^3J_{HH}$ = 6.8 Hz), 7.20 – 7.47 (m, 7H), 6.20 (d, 1H, $^3J_{HH}$ = 4.2 Hz), 6.03 (s, 1H), 5.22 (t, 1H, $^3J_{HH}$ = 5.3 Hz), 4.53 (m, 1H), 4.39 – 4.45 (m, 2H), 4.11 – 4.19 (m, 3H), 3.98 (m, 1H), 3.42 (m, 8H), 3.01 (dd, 1H, $^3J_{HH}$ = 3.9 Hz, $^2J_{HH}$ = 13.5 Hz), 2.80 (m, 1H). $^{19}$F NMR (376 MHz, DMSO-d6): -119.53 (d, 1F, $^3J_{FF}$ = 22.5 Hz), -142.24 (d, 1F, $^3J_{FF}$ = 22.5 Hz). $^{13}$C NMR (125 MHz, DMSO-d6): 171.59, 155.74, 154.24, 151.85, 149.63, 148.92 (dd, $^2J_{CF}$ = 12.8 Hz, $^1J_{CF}$ = 244.9 Hz), 148.29 (dd, $^2J_{CF}$ = 12.6 Hz, $^1J_{CF}$ = 244.7 Hz), 143.75, 143.64, 140.65, 140.62, 137.88, 137.91 (m), 127.60, 126.96, 126.13 (m), 125.27, 125.19, 120.08, 119.60, 118.17 (d, $^2J_{CF}$ = 16.9 Hz), 116.85 (d, $^2J_{CF}$ = 16.8 Hz), 89.42, 83.41, 73.04, 65.66, 60.87, 55.78, 50.29, 46.48, 37.03. ESI-MS ($ES^+$): m/z calcd for $C_{36}H_{35}F_2N_7O_6$ 699.2, found 700.2 ($MH^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-2-trifluoromethylphenylalanyl]-$N^6$,$N^6$-dimethyladenosine* **2f**. Isolated in 98% yield (490 mg) by flash chromatography ($CH_2Cl_2$ /MeOH, 95:5), starting from puromycin aminonucleoside (200 mg, 0.680 mmol), acyl fluoride **1f** (314 mg, 0.686 mmol), anhydrous pyridine (1 ml), anhydrous DMF (19 ml). $^1$H NMR (400 MHz, DMSO-d6): 8.46 (s, 1H), 8.24 (s, 1H), 8.05 (d, 1H, $^3J_{HH}$ = 7.3 Hz), 7.90 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.81 (d, 1H, $^3J_{HH}$ = 8.9 Hz), 7.69 (d, 3H, $^3J_{HH}$ = 7.4 Hz), 7.30 – 7.55 (m, 7H), 6.07 (d, 1H, $^3J_{HH}$ = 4.5 Hz), 6.00 (d, 1H, $^3J_{HH}$ = 2.6 Hz), 5.22 (t, 1H, $^3J_{HH}$ = 5.1 Hz), 4.45 – 4.54 (m, 3H), 4.15 – 4.21 (m, 3H), 3.95 (m, 1H), 3.41 – 3.69 (m, 8H), 3.28 (dd, 1H, $^3J_{HH}$ = 4.5 Hz, $^2J_{HH}$ = 14.0 Hz), 3.01 (dd, 1H, $^3J_{HH}$ = 9.9 Hz, $^2J_{HH}$ = 13.7 Hz). $^{19}$F NMR (376 MHz, DMSO-d6): -57.99 (s, 3F). $^{13}$C NMR (100 MHz, DMSO-d6): 171.10, 155.66, 154.24, 151.84, 149.65, 143.72, 143.69, 140.65, 137.90, 136.08,

132.04, 131.70, 127.64, 127.49 (q, $^{2}J_{CF}$ = 29.1 Hz), 127.04, 125.79 (q, $^{3}J_{CF}$ = 5.1 Hz), 125.28, 124.55 (q, $^{1}J_{CF}$ = 273.7 Hz), 120.10, 119.58, 89.22, 83.23, 73.00, 65.82, 60.85, 55.03, 50.35, 46.48, 34.31. ESI-MS ($ES^{+}$): m/z calcd for $C_{37}H_{36}F_{3}N_{7}O_{6}$ 731.2, found 732.3 ($MH^{+}$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-pentafluorophenylalanyl]-$N^{6}$,$N^{6}$-dimethyladenosine* **2g**. Isolated in 87% yield (450 mg) by flash chromatography ($CH_2Cl_2$ /MeOH, 95:5), starting from puromycin aminonucleoside (200 mg, 0.680 mmol), acyl fluoride **1g** (328 mg, 0.686 mmol), anhydrous pyridine (1 ml), anhydrous DMF (19 ml). $^{1}$H NMR (400 MHz, DMSO-d6): 8.44 (s, 1H), 8.28 (d, 1H, $^{3}J_{HH}$ = 7.6 Hz), 8.24 (s, 1H), 7.89 (d, 2H, $^{3}J_{HH}$ = 7.5 Hz), 7.65 – 7.76 (m, 3H), 7.40 – 7.44 (m, 2H), 7.32 (t, 2H, $^{3}J_{HH}$ = 7.4 Hz), 5.96 (d, 1H, $^{3}J_{HH}$ = 3.3 Hz), 5.94 (d, 1H, $^{3}J_{HH}$ = 4.9 Hz), 5.22 (t, 1H, $^{3}J_{HH}$ = 5.3 Hz), 4.41 – 4.53 (m, 3H), 4.27 – 4.33 (m, 1H), 4.14 – 4.19 (m, 2H), 3.93 – 3.95 (m, 1H), 3.42 – 4.69 (m, 8H), 3.17 (dd, 1H, $^{3}J_{HH}$ = 6.9 Hz, $^{2}J_{HH}$ = 13.9 Hz), 2.99 (dd, 1H, $^{3}J_{HH}$ = 7.3 Hz, $^{2}J_{HH}$ = 13.5 Hz). $^{19}$F NMR (376 MHz, DMSO-d6): -141.50 (dd, 2F, $^{4}J_{FF}$ = 7.4 Hz, $^{3}J_{FF}$ = 23.9 Hz), -157.45 (t, 1F, $^{3}J_{FF}$ = 22.2 Hz), -163.41 (dt, 2F, $^{4}J_{FF}$ = 6.7 Hz, $^{3}J_{FF}$ = 23.2 Hz). $^{13}$C NMR (100 MHz, DMSO-d6): 172.77, 169.97, 155.56, 154.23, 151.84, 149.69, 146.36 (m), 143.93 (m), 143.69, 143.65, 140.67, 140.64, 137.80, 135.36 (m), 127.64, 127.61, 127.02, 126.99, 125.27, 125.21, 120.09, 120.06, 119.57, 111.27 (m), 88.97, 82.92, 72.96, 65.92, 60.77, 53.27, 50.55, 46.48, 25.83, 25.21. ESI-MS ($ES^{+}$): m/z calcd for $C_{36}H_{32}F_{5}N_{7}O_{6}$ 753.2, found 754.5 ($MH^{+}$).

**General Procedure for the Preparation of Puromycin Derivatives 2h-j (Method 2)**
After co-evaporationing with pyridine (3 x 10ml), puromycin aminonucleoside (1 eq) was placed in a 50 ml round bottom flask under argon. Anhydrous DMF, N-(9-fluorenylmethoxycarbonyl)-amino acid (1.1 eq) and N-hydroxysuccinimide (SuOH, 1.1 eq) were introduced and the solution was cooled to 0°C. 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI, 1 eq) was added and the solution was stirred 24 hours at room temperature. After evaporation in vacuo, the oily residue was purified by flash chromatography to produce puromycin derivatives **2h-j**.

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-4-methylphenylalanyl]-$N^6$,$N^6$-dimethyladenosine* **2h**. Isolated in 87% yield (400 mg) by flash chromatography ($CH_2Cl_2$ /MeOH, 95:5), starting from puromycin aminonucleoside (200 mg, 0.680 mmol), N-(9-fluorenylmethoxycarbonyl)-4-methylphenylalanine (300 mg, 0.748 mmol), SuOH (86 mg, 0.748 mmol), EDCI (130 mg, 0.680 mmol), anhydrous DMF (9 ml). $^1$H NMR (400 MHz, DMSO-d6): 8.46 (s, 1H), 8.25 (s, 1H), 8.20 (d, 1H, $^3J_{HH}$ = 7.1 Hz), 7.88 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.64 (m, 2H), 7.41 (t, 2H, $^3J_{HH}$ = 7.4 Hz), 7.31 (m, 3H), 7.23 (d, 2H, $^3J_{HH}$ = 7.7 Hz), 7.07 (d, 2H, $^3J_{HH}$ = 7.7 Hz), 6.13 (d, 1H, $^3J_{HH}$ = 4.4 Hz), 6.01 (d, 1H, $^3J_{HH}$ = 1.9 Hz), 5.21 (t, 1H, $^3J_{HH}$ = 5.3 Hz), 4.50 (m, 2H), 4.37 (m, 1H), 4.11 – 4.21 (m, 3H), 3.96 (m, 1H), 3.47 – 3.71 (m, 8H), 2.97 (dd, 1H, $^3J_{HH}$ = 4.1 Hz, $^2J_{HH}$ = 13.4 Hz), 2.77 (dd, 1H, $^3J_{HH}$ = 10.5 Hz, $^2J_{HH}$ = 12.0 Hz), 2.24 (s, 3H). $^{13}$C NMR (100 MHz, DMSO-d6): 171.96, 155.70, 154.24, 151.85, 149.64, 143.75, 140.62, 137.88, 135.09, 134.91, 129.20, 128.58, 127.59, 127.01, 125.35, 125.26, 120.06, 119.60, 89.36, 83.38, 73.02, 65.63, 60.87, 56.14, 50.26, 46.51, 37.50. ESI-MS ($ES^+$): m/z calcd for $C_{37}H_{39}N_7O_6$ 677.3, found 678.6 ($MH^+$), 700.4 ($M + Na^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-4-(N-terbutylmethoxycarbonyl)-phenylalanyl]-$N^6$,$N^6$-dimethyladenosine* **2i**. Isolated in 85% yield (450 mg) by flash chromatography ($CH_2Cl_2$ /MeOH, 95:5), starting from puromycin aminonucleoside (200 mg, 0.680 mmol), N-(9-fluorenylmethoxycarbonyl)-4-(N-terbutylmethoxycarbonyl)-phenylalanine (386 mg, 0.748 mmol), SuOH (86 mg, 0.748 mmol), EDCI (130 mg, 0.680 mmol), anhydrous DMF (9 ml). $^1$H NMR (500 MHz, DMSO-d6): 9.23 (sbr, 1H), 8.45 (s, 1H), 8.24 (s, 1H), 8.14 (d, 1H, $^3J_{HH}$ = 7.1 Hz), 7.87 (d, 2H, $^3J_{HH}$ = 7.4 Hz), 7.64 (m, 2H), 7.55 (d, 1H, $^3J_{HH}$ = 8.7 Hz), 7.28 – 7.40 (m, 6H), 7.21 (d, 2H, $^3J_{HH}$ = 8.0 Hz), 6.10 (d, 1H, $^3J_{HH}$ = 4.1 Hz), 6.01 (sbr, 1H), 5.18 (t, 1H, $^3J_{HH}$ = 5.0 Hz), 4.48 – 4.52 (m, 2H), 4.32 – 4.37 (m, 1H), 4.10 – 4.22 (m, 3H), 3.97 (m, 1H), 3.37 – 3.71 (m, 8H), 2.95 (dd, 1H, $^3J_{HH}$ = 3.4 Hz, $^2J_{HH}$ = 13.4 Hz), 2.74 (m, 1H), 1.47 (s, 9H). $^{13}$C NMR (125 MHz, DMSO-d6): 172.29, 162.66, 156.06, 154.67, 153.16, 152.22, 150.06, 144.17, 144.11, 141.02, 138.27, 138.15, 131.88, 129.82, 127.93, 127.43, 127.41, 125.71, 125.59, 120.42, 120.03, 118.22, 89.75, 83.83, 79.20, 73.42, 66.05, 61.33, 56.60, 50.73, 46.93, 37.66, 36.14, 28.51. ESI-MS ($ES^+$): m/z calcd for $C_{41}H_{46}N_8O_8$ 778.3, found 779.2 ($MH^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-4-trifluoromethylphenylalanyl]-$N^6$,$N^6$-dimethyladenosine* **2j**. Isolated in 85% yield (422 mg) by flash chromatography ($CH_2Cl_2$ /MeOH, 95:5), starting from puromycin aminonucleoside (200 mg, 0.680 mmol), N-(9-fluorenylmethoxycarbonyl)-4-trifluoromethylphenylalanine (340 mg, 0.748 mmol), SuOH (86 mg, 0.748 mmol), EDCI (130 mg, 0.680 mmol), anhydrous DMF (9 ml). $^1$H NMR (400 MHz, DMSO-d6): 8.47 (s, 1H), 8.33 (d, 1H, $^3J_{HH}$ = 6.6 Hz), 8.25 (s, 1H), 7.88 (d, 2H, $^3J_{HH}$ = 7.4 Hz), 7.72 (d, 1H, $^3J_{HH}$ = 8.7 Hz), 7.57 – 7.64 (m, 6H), 7.25 – 7.50 (m, 4H), 6.20 (sbr, 1H), 6.02 (s, 1H), 5.77 (s, 1H), 5.22 (m, 1H), 4.44 (m, 3H), 4.11 – 4.19 (m, 2H), 3.97 (m, 1H), 3.48 – 3.71 (m, 8H), 3.10 – 3.28 (m, 1H), 2.88 – 2.94 (m, 1H). $^{19}$F NMR (376 MHz, DMSO-d6): -60.75 (s, 3F). $^{13}$C NMR (100 MHz, DMSO-d6): 172.79, 171.57, 155.73, 154.24, 151.85, 149.93, 143.73, 143.67, 143.04, 140.62, 137.88, 130.15, 127.56, 126.97, 125.28, 124.81, 124.78, 124.41 (q, $^1J_{CF}$ = 270.1 Hz), 120.07, 119.60, 89.39, 83.36, 73.03, 65.65, 60.85, 55.64, 54.91, 50.31, 46.58, 37.68. ESI-MS ($ES^+$): m/z calcd for $C_{37}H_{36}F_3N_7O_6$ 731.2, found 732.4 ($MH^+$).

**General Procedure for the Preparation of 5'-Protected Puromycin Derivatives 3a-j**

In a 25 ml round bottom flask under argon, anhydrous pyridine and anhydrous triethylamine (2.8 eq) were added to puromycin derivatives **2a-j** (1 eq). Dimethoxytrityl chloride (DmTrCl, 2.5 eq) was added and the solution was stirred at room temperature for 3 hours. Methanol (10 ml) was added to quench the reaction and the solution was stirred at room temperature for 2 hours. After evaporation in vacuo, the residue was purified by flash chromatography to produce the corresponding 5'-protected puromycin derivatives **3a-j**.

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-3,4-dichlorophenylalanyl]-5'-O-(p,p'dimethoxytrityl)-$N^6$,$N^6$-dimethyladenosine* **3a**. Isolated in 72% yield (250 mg) by flash chromatography ($CH_2Cl_2$/ MeOH, 96:4), starting from puromycin derivative **2a** (245 mg, 0.335 mmol), DmTrCl (298 mg, 0.837 mmol), anhydrous $NEt_3$ (130 µl, 0.938 mmol), anhydrous pyridine (10 ml). $^1$H NMR (500 MHz, DMSO-d6): 8.35 (s, 1H), 8.32 (d, 1H, $^3J_{HH}$ = 8.7 Hz), 8.28 (s, 1H), 7.92 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.69 – 7.72 (m, 2H), 7.66 (d, 1H, $^3J_{HH}$ = 7.5 Hz), 7.63 (d, 1H, $^3J_{HH}$ = 7.5 Hz), 7.55 (d, 1H, $^3J_{HH}$ = 8.1 Hz), 7.23 – 7.46 (m, 14 H), 6.83 – 6.88 (m, 4H), 6.38 (d, 1H, $^3J_{HH}$ = 4.8 Hz), 6.10 (sbr, 1H), 4.82 – 4.87

(m, 1H), 4.65 – 4.67 (m, 1H), 4.36 – 4.45 (m, 1H), 4.13 – 4.20 (m, 4H), 3.71 (s, 3H), 3.70 (s, 3H), 3.56 (sbr, 6H), 3.32 (m, 1H), 3.17 (dd, 1H, $^3J_{HH}$ = 5.3 Hz, $^2J_{HH}$ = 10.8 Hz), 2.73 – 2.78 (m, 1H), 2.56 – 2.63 (m, 1H). $^{13}$C NMR (100 MHz, DMSO-d6): 172.06, 158.36, 156.13, 154.62, 152.38, 150.05, 145.11, 144.14, 144.00, 141.02, 140.98, 139.88, 138.07, 135.67, 135.61, 131.64, 130.85, 130.42, 130.12, 129.29, 128.15, 128.03, 127.97, 127.35, 126.97, 125.65, 125.56, 120.46, 120.06, 113.57, 113.45, 90.45, 85.89, 81.28, 73.06, 66.01, 63.11, 60.12, 56.06, 55.24, 50.69, 46.84, 37.51. ESI-MS ($ES^+$): m/z calcd for $C_{57}H_{53}Cl_2N_7O_8$ 1033.3, found 1056.8 (M + $Na^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-2,4-dichlorophenylalanyl]-5'-O-(p,p'dimethoxytrityl)-N$^6$,N$^6$-dimethyladenosine* **3b**. Isolated in 72% yield (250 mg) by flash chromatography ($CH_2Cl_2$/ MeOH, 96:4), starting from puromycin derivative **3a** (245 mg, 0.335 mmol), DmTrCl (298 mg, 0.837 mmol), anhydrous $NEt_3$ (130 µl, 0.938 mmol), anhydrous pyridine (10 ml). $^1$H NMR (400 MHz, DMSO-d6): 8.17 (s, 1H), 8.11 (s, 1H), 7.94 (d, 1H, $^3J_{HH}$ = 8.3 Hz), 7.76 (d, 2H, $^3J_{HH}$ = 7.4 Hz), 7.49 – 7.54 (m, 2H), 7.41 (d, 1H, $^4J_{HH}$ = 2.0 Hz), 7.04 – 7.30 (m, 16 H), 6.69 (d, 2H, $^3J_{HH}$ = 5.5 Hz), 6.66 (d, 2H, $^3J_{HH}$ = 5.5 Hz), 6.01 (d, 1H, $^3J_{HH}$ = 4.9 Hz), 5.91 (d, 1H, $^3J_{HH}$ = 2.3 Hz), 4.62 – 4.68 (m, 1H), 4.53 – 4.56 (m, 1H), 4.31 – 4.37 (m, 1H), 3.96 – 4.06 (m, 4H), 3.56 (s, 6H), 3.38 (sbr, 6H), 3.14 (m, 1H), 3.04 (dd, 1H, $^3J_{HH}$ = 5.0 Hz, $^2J_{HH}$ = 10.5 Hz), 2.87 (dd, 1H, $^3J_{HH}$ = 4.6 Hz, $^2J_{HH}$ = 14.1 Hz), 2.73 (dd, 1H, $^3J_{HH}$ = 10.4 Hz, $^2J_{HH}$ = 14.0 Hz). $^{13}$C NMR (100 MHz, DMSO-d6): 171.38, 158.37, 158.35, 156.04, 154.64, 152.35, 150.15, 145.11, 144.12, 144.03, 141.04, 141.01, 138.14, 135.80, 135.69, 134.81, 133.03, 132.22, 130.09, 130.03, 128.86, 128.10, 128.07, 127.98, 127.38, 127.26, 126.97, 125.65, 125.60, 120.46, 120.07, 113.43, 90.00, 85.87, 81.28, 72.96, 66.16, 63.30, 60.12, 55.27, 54.19, 50.92, 46.85, 35.47. ESI-MS ($ES^+$): m/z calcd for $C_{57}H_{53}Cl_2N_7O_8$ 1033.3, found 1056.7 (M + $Na^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-2-nitrophenylalanyl]-5'-O-(p,p'dimethoxytrityl)-N$^6$,N$^6$-dimethyladenosine* **3c**. Isolated in 72% yield (270 mg) by flash chromatography (AcOEt/Hex, 8:2), starting from puromycin derivative **2c** (260 mg, 0.366 mmol), DmTrCl (330 mg, 0.915 mmol), anhydrous $NEt_3$ (150 µl, 1.024 mmol), anhydrous pyridine (12 ml). $^1$H NMR (400 MHz, DMSO-d6): 8.30 (s, 1H), 8.24 (s, 1H),

8.03 (d, 1H, $^3J_{HH}$ = 8.2 Hz), 7.97 (d, 1H, $^3J_{HH}$ = 7.9 Hz), 7.88 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.70 (d, 1H, $^3J_{HH}$ = 9.0 Hz), 7.63 (d, 2H, $^3J_{HH}$ = 7.4 Hz), 7.49 (d, 2H, $^4J_{HH}$ = 4.0 Hz), 7.17 – 7.45 (m, 14H), 6.82 (d, 2H, $^3J_{HH}$ = 8.7 Hz), 6.81 (d, 2H, $^3J_{HH}$ = 8.8 Hz), 6.15 (d, 1H, $^3J_{HH}$ = 4.7 Hz), 6.03 (d, 1H, $^3J_{HH}$ = 2.3 Hz), 4.73 – 4.79 (m, 1H), 4.67 – 4.70 (m, 1H), 4.53 – 4.59 (m, 1H), 4.10 – 4.16 (m, 4H), 3.69 (s, 3H), 3.68 (s, 3H), 3.46 (sbr, 6H), 3.16 – 3.26 (m, 3H), 3.05 (dd, 1H, $^3J_{HH}$ = 10.2 Hz, $^2J_{HH}$ = 13.9 Hz). $^{13}$C NMR (100 MHz, DMSO-d6): 171.00, 157.98, 157.96, 155.68, 154.24, 151.97, 149.76, 149.24, 144.78, 143.66, 140.64, 140.61, 137.79, 135.43, 135.36, 132.95, 132.46, 132.35, 129.69, 129.65, 127.95, 127.73, 127.68, 127.61, 127.03, 126.58, 125.30, 125.21, 124.58, 120.07, 119.67, 113.05, 89.53, 85.50, 81.02, 72.57, 65.77, 63.03, 54.87, 54.49, 50.53, 46.44, 34.60. ESI-MS ($ES^+$): m/z calcd for $C_{57}H_{54}N_8O_{10}$ 1010.4, found 1011.5 ($MH^+$), 1033.1 (M + $Na^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-2-fluorophenylalanyl]-5'-O-(p,p'dimethoxytrityl)-N$^6$,N$^6$-dimethyladenosine* **3d**. Isolated in 72% yield (300 mg) by flash chromatography ($CH_2Cl_2$/ MeOH, 97:3), starting from puromycin derivative **2d** (260 mg, 0.381 mmol), DmTrCl (408 mg, 1.143 mmol), anhydrous $NEt_3$ (180 µl, 1.257 mmol), anhydrous pyridine (11 ml). $^1$H NMR (500 MHz, $CDCl_3$): 8.29 (s, 1H), 8.23 (s, 1H), 8.12 (d, 1H, $^3J_{HH}$ = 8.3 Hz), 7.88 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.58 – 7.65 (m, 3H), 7.10 – 7.42 (m, 14H), 6.98 – 7.12 (m, 3H), 6.82 (d, 2H, $^3J_{HH}$ = 8.7 Hz), 6.80 (d, 2H, $^3J_{HH}$ = 8.7 Hz), 6.13 (d, 1H, $^3J_{HH}$ = 4.7 Hz), 6.03 (d, 1H, $^3J_{HH}$ = 2.2 Hz), 4.73 – 4.79 (m, 1H), 4.64 – 4.67 (m, 1H), 4.42 – 4.48 (m, 1H), 4.09 – 4.19 (m, 4H), 3.68 (sbr, 6H), 3.48 (sbr, 6H), 3.25 (m, 1H), 3.16 (dd, 1H, $^3J_{HH}$ = 5.0 Hz, $^2J_{HH}$ = 10.3 Hz), 2.93 (dd, 1H, $^3J_{HH}$ = 4.4 Hz, $^2J_{HH}$ = 14.1 Hz), 2.78 (m, 1H). $^{19}$F NMR (376 MHz, $CDCl_3$): -117.04 (s, 1F). $^{13}$C NMR (125 MHz, $CDCl_3$): 171.25, 161.27 (d, $^1J_{CF}$ = 245.1 Hz), 158.50, 155.96, 154.91, 151.66, 149.76, 149.15, 144.41, 143.77, 143.65, 141.27, 141.24, 136.02, 135.70, 135.60, 131.60 (d, $^3J_{CF}$ = 3.8 Hz), 130.06, 129.94, 129.47, 129.17, 128.95 (d, $^3J_{CF}$ = 8.8 Hz), 128.18, 127.83, 127.72, 127.07, 126.84, 125.08, 124.37 (d, $^4J_{CF}$ = 2.5 Hz), 123.54 (d, $^2J_{CF}$ = 15.1 Hz), 120.57, 119.97, 119.95, 115.40 (d, $^2J_{CF}$ = 22.6 Hz), 113.19, 91.29, 86.49, 84.10, 74.54, 67.24, 63.58, 55.35, 55.25, 53.41, 52.69, 47.02, 32.30. ESI-MS ($ES^+$): m/z calcd for $C_{57}H_{54}FN_7O_8$ 984.4, found 1006.8 (M + $Na^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-3,4-difluorophenylalanyl]-5'-O-(p,p'dimethoxytrityl)-$N^6$,$N^6$-dimethyladenosine* **3e**. Isolated in 78% yield (280 mg) by flash chromatography (AcOEt/Hex, 7:3), starting from puromycin derivative **2e** (250 mg, 0.357 mmol), DmTrCl (320 mg, 0.893 mmol), anhydrous $NEt_3$ (140 µl, 0.999 mmol), anhydrous pyridine (12 ml). $^1$H NMR (400 MHz, DMSO-d6): 8.32 (s, 1H), 8.27 (d, 1H, $^3J_{HH}$ = 8.7 Hz), 8.24 (s, 1H), 7.88 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.67 (d, 1H, $^3J_{HH}$ = 9.0 Hz), 7.61 (t, 2H, $^3J_{HH}$ = 7.5 Hz), 7.17 – 7.44 (m, 16H), 6.83 (d, 2H, $^3J_{HH}$ = 8.9 Hz), 6.82 (d, 2H, $^3J_{HH}$ = 8.9 Hz), 6.36 (d, 1H, $^3J_{HH}$ = 4.8 Hz), 6.06 (sbr, 1H), 4.77 – 4.83 (m, 1H), 4.61 – 4.63 (m, 1H), 4.31 – 4.37 (m, 1H), 4.05 – 4.17 (m, 4H), 3.67 (s, 3H), 3.66 (s, 3H), 3.48 (sbr, 6H), 3.29 – 3.32 (m, 1H), 3.12 (dd, 1H, $^3J_{HH}$ = 5.1 Hz, $^2J_{HH}$ = 10.6 Hz), 2.52 – 2.72 (m, 2H). $^{19}$F NMR (376 MHz, DMSO-d6): -139.60 (d, 1F, $^3J_{FF}$ = 22.5 Hz), -142.29 (d, 1F, $^3J_{FF}$ = 22.6 Hz). $^{13}$C NMR (100 MHz, DMSO-d6): 171.71, 157.98, 155.74, 154.24, 151.99, 150.16 (m), 147.60 (m), 144.73, 143.75, 143.63, 140.64, 140.60, 137.68, 135.99 (m), 135.30, 135.23, 129.73, 127.76, 127.66, 127.60, 126.98, 126.93, 126.59, 126.10 (m), 125.27, 125.18, 120.08, 118.68, 118.10 (d, $^2J_{CF}$ = 16.7 Hz), 116.80 (d, $^2J_{CF}$ = 16.7 Hz), 113.08, 90.04, 85.50, 80.90, 72.69, 65.61, 62.74, 55.79, 54.85, 50.29, 46.46, 37.19. ESI-MS ($ES^+$): m/z calcd for $C_{57}H_{53}F_2N_7O_8$ 1001.4, found 1002.4 ($MH^+$), 1024.3 ($M + Na^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-2-trifluoromethylphenylalanyl]-5'-O-(p,p'dimethoxytrityl)-$N^6$,$N^6$-dimethyladenosine* **3f**. Isolated in 73% yield (280 mg) by flash chromatography (AcOEt/Hex, 7:3), starting from puromycin derivative **2f** (270 mg, 0.368 mmol), DmTrCl (330 mg, 0.920 mmol), anhydrous $NEt_3$ (150 µl, 1.030 mmol), anhydrous pyridine (12 ml). $^1$H NMR (400 MHz, DMSO-d6): 8.30 (s, 1H), 8.24 (s, 1H), 8.03 (d, 1H, $^3J_{HH}$ = 8.1 Hz), 7.89 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.75 (d, 1H, $^3J_{HH}$ = 9.1 Hz), 7.65 – 7.67 (m, 3H), 7.16 – 7.50 (m, 16 H), 6.81 (d, 2H, $^3J_{HH}$ = 8.5 Hz), 6.79 (d, 2H, $^3J_{HH}$ = 8.6 Hz), 6.13 (d, 1H, $^3J_{HH}$ = 4.8 Hz), 6.04 (d, 1H, $^3J_{HH}$ = 2.3 Hz), 4.69 – 4.81 (m, 2H), 4.43 – 4.49 (m, 1H), 4.08 – 4.16 (m, 4H), 3.69 (s, 6H), 3.48 (sbr, 6H), 3.16 – 3.24 (m, 3H), 2.94 (dd, 1H, $^3J_{HH}$ = 10.2 Hz, $^2J_{HH}$ = 13.8 Hz). $^{19}$F NMR (376 MHz, DMSO-d6): -57.95 (s, 3F). $^{13}$C NMR (100 MHz, DMSO-d6): 171.01, 157.97, 157.94, 155.65, 154.24, 151.96, 149.78, 144.76, 143.71, 143.65, 140.65, 140.62, 137.83, 136.08, 135.43, 135.35, 131.90, 131.74, 129.68, 129.61, 127.66, 127.63, 127.36, 126.78 (q, $^2J_{CF}$ = 35.2

Hz), 125.73 (m), 125.24, 124.51 (q, $^{1}J_{CF}$ = 273.8 Hz), 120.09, 119.67, 113.01, 89.51, 85.46, 81.00, 72.55, 65.81, 63.04, 55.03, 54.84, 50.60, 46.45, 34.39. ESI-MS ($ES^{+}$): m/z calcd for $C_{58}H_{54}F_3N_7O_8$ 1033.4, found 1034.4 ($MH^{+}$), 1056.3 (M + $Na^{+}$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-pentafluorophenylalanyl]-5'-O-(p,p'dimethoxytrityl)-$N^6$,$N^6$-dimethyladenosine* **3g**. Isolated in 40% yield (113 mg) by flash chromatography ($CH_2Cl_2$ /MeOH, 97:3), starting from puromycin derivative **2g** (210 mg, 0.278 mmol), DmTrCl (300 mg, 0.835 mmol), anhydrous $NEt_3$ (130 µl, 0.917 mmol), anhydrous pyridine (8.5 ml). $^{1}$H NMR (400 MHz, DMSO-d6): 8.38 (sbr, 1H), 8.35 (s, 1H), 8.30 (s, 1H), 7.95 – 7.97 (m, 3H), 7.72 – 7.75 (m, 3H), 7.25 – 7.49 (m, 10 H), 7.06 – 7.16 (m, 2H), 6.89 (d, 2H, $^{3}J_{HH}$ = 8.7 Hz), 6.88 (d, 2H, $^{3}J_{HH}$ = 8.7 Hz), 6.09 (d, 1H, $^{3}J_{HH}$ = 1.9 Hz), 6.08 (d, 1H, $^{3}J_{HH}$ = 4.0 Hz), 4.58 (m, 1H), 4.12 – 4.34 (m, 6H), 3.76 (s, 6H), 3.48 (sbr, 6H), 3.25 – 3.37 (m, 2H), 3.17 (dd, 1H, $^{3}J_{HH}$ = 5.4 Hz, $^{2}J_{HH}$ = 14.1 Hz), 2.98 (dd, 1H, $^{3}J_{HH}$ = 8.7 Hz, $^{2}J_{HH}$ = 14.0 Hz). $^{19}$F NMR (376 MHz, DMSO-d6): -141.46 (dd, 2F, $^{4}J_{FF}$ = 7.5 Hz, $^{3}J_{FF}$ = 24.2 Hz), -157.36 (t, 1F, $^{3}J_{FF}$ = 21.6 Hz), -163.44 (dt, 2F, $^{4}J_{FF}$ = 7.2 Hz, 3JFF = 22.7 Hz). $^{13}$C NMR (100 MHz, DMSO-d6): 169.91, 157.97, 157.84, 155.65, 154.26, 151.96, 149.82, 144.90, 144.71, 143.96 (m), 143.65, 143.61, 143.56, 140.66, 140.61, 137.80, 135.44, 135.37, 135.19 (m), 129.70, 129.63, 127.69, 127.64, 127.60, 127.48, 120.05, 119.69, 113.02, 112.82, 89.34, 85.52, 80.78, 72.44, 65.92, 63.13, 54.88, 53.16, 51.02, 46.46. ESI-MS ($ES^{+}$): m/z calcd for $C_{57}H_{50}F_5N_7O_8$ 1055.4, found 1056.9 ($MH^{+}$), 1078.5 (M + $Na^{+}$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-4-methylphenylalanyl]-5'-O-(p,p'dimethoxytrityl)-$N^6$,$N^6$-dimethyladenosine* **3h**. Isolated in 79% yield (240 mg) by flash chromatography (AcOEt/Hex, 7:3), starting from puromycin derivative **2h** (210 mg, 0.309 mmol), DmTrCl (280 mg, 0.772 mmol), anhydrous $NEt_3$ (120 µl, 0.865 mmol), anhydrous pyridine (10 ml). $^{1}$H NMR (500 MHz, DMSO-d6): 8.30 (s, 1H), 8.24 (s, 1H), 8.17 (d, 1H, $^{3}J_{HH}$ = 8.5 Hz), 7.87 (d, 2H, $^{3}J_{HH}$ = 7.5 Hz), 7.64 (d, 1H, $^{3}J_{HH}$ = 7.5 Hz), 7.62 (d, 1H, $^{3}J_{HH}$ = 7.5 Hz), 7.56 (d, 1H, $^{3}J_{HH}$ = 8.8 Hz), 7.17 – 7.42 (m, 15H), 7.03 (d, 2H, $^{3}J_{HH}$ = 8.4 Hz), 6.80 – 6.83 (m, 4H), 6.22 (d, 1H, $^{3}J_{HH}$ = 4.8 Hz), 6.05 (d, 1H, $^{3}J_{HH}$ = 1.8 Hz), 4.76 – 4.81 (m, 1H), 4.62 – 4.64 (m, 1H), 4.31 – 4.36 (m, 1H), 4.08 – 4.17 (m, 4H), 3.67 (s, 3H), 3.66 (s, 3H), 3.38 (sbr, 6H), 3.30 (m, 1H), 3.14 (dd, 1H, $^{3}J_{HH}$ = 5.2 Hz, $^{2}J_{HH}$

= 10.7 Hz), 2.76 (dd, 1H, $^3J_{HH}$ = 3.7 Hz, $^2J_{HH}$ = 13.6 Hz), 2.63 (m, 1H), 2.23 (s, 3H). $^{13}$C NMR (125 MHz, DMSO-d6): 172.37, 158.40, 158.38, 156.09, 154.67, 152.35, 150.13, 145.14, 144.15, 144.12, 141.02, 141.00, 138.03, 135.83, 135.70, 135.39, 135.36, 130.11, 130.08, 129.53, 128.90, 128.11, 128.09, 127.95, 127.38, 126.97, 125.72, 125.62, 120.41, 120.11, 113.48, 113.46, 90.25, 85.90, 81.34, 73.07, 66.01, 63.25, 56.49, 55.27, 50.77, 46.92, 38.05, 21.02. ESI-MS ($ES^+$): m/z calcd for $C_{58}H_{57}N_7O_8$ 979.4, found 980.7 ($MH^+$), 1002.8 ($M + Na^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl-L-4-(N-terbutylmethoxycarbonyl)-phenylalanyl]-5'-O-(p,p'dimethoxytrityl)-N$^6$,N$^6$-dimethyladenosine* **3i**. Isolated in 67% yield (280 mg) by flash chromatography (AcOEt/Hex, 7:3), starting from puromycin derivative **2i** (300 mg, 0.385 mmol), DmTrCl (343 mg, 0.962 mmol), anhydrous $NEt_3$ (150 µl, 1.078 mmol), anhydrous pyridine (13 ml). $^1$H NMR (400 MHz, DMSO-d6): 9.26 (s, 1H), 8.31 (s, 1H), 8.24 (s, 1H), 8.22 (d, 1H, $^3J_{HH}$ = 8.5 Hz), 7.88 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.65 (d, 1H, $^3J_{HH}$ = 7.4 Hz), 7.59 (t, 2H, $^3J_{HH}$ = 7.1 Hz), 7.18 – 7.44 (m, 17H), 6.82 (d, 2H, $^3J_{HH}$ = 8.8 Hz), 6.81 (d, 2H, $^3J_{HH}$ = 8.8 Hz), 6.29 (d, 1H, $^3J_{HH}$ = 4.8 Hz), 6.05 (sbr, 1H), 4.77 – 4.82 (m, 1H), 4.62 (m, 1H), 4.28 – 4.34 (m, 1H), 4.06 – 4.18 (m, 4H), 3.67 (s, 3H), 3.66 (s, 3H), 3.48 (sbr, 6H), 3.30 (m, 1H), 3.11 – 3.15 (m, 1H), 2.54 – 2.73 (m, 2H), 1.4 (s, 9H). $^{13}$C NMR (100 MHz, DMSO-d6): 172.06, 157.97, 155.68, 154.23, 152.76, 151.99, 149.68, 144.74, 143.78, 143.71, 140.60, 137.69, 137.64, 135.69, 135.25, 131.64, 129.72, 129.42, 127.76, 127.67, 127.54, 127.01, 126.58, 125.36, 125.23, 120.04, 119.68, 117.73, 89.92, 85.49, 80.87, 78.80, 72.68, 65.59, 62.71, 56.15, 54.91, 54.87, 50.27, 46.49, 45.64, 37.46, 28.11. ESI-MS ($ES^+$): m/z calcd for $C_{62}H_{64}N_8O_{10}$ 1080.4, found 1081.5 ($MH^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-4-trifluoromethylphenylalanyl]-5'-O-(p,p'dimethoxytrityl)-N$^6$,N$^6$-dimethyladenosine* **3j**. Isolated in 54% yield (190 mg) by flash chromatography (AcOEt/Hex, 7:3), starting from puromycin derivative **2j** (250 mg, 0.341 mmol), DmTrCl (305 mg, 0.852 mmol), anhydrous $NEt_3$ (133 µl, 0.954 mmol), anhydrous pyridine (11 ml). $^1$H NMR (400 MHz, DMSO-d6): 8.34 (sbr, 1H), 8.32 (s, 1H), 8.23 (s, 1H), 7.87 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.70 (d, 1H, $^3J_{HH}$ = 9.0 Hz), 7.54 – 7.63 (m, 6H), 7.17 – 7.44 (m, 13H), 6.82 (d, 2H, $^3J_{HH}$ = 8.9 Hz), 6.81 (d, 2H, $^3J_{HH}$ = 8.9 Hz), 6.34

(d, 1H, $^{3}J_{HH}$ = 4.8 Hz), 6.06 (sbr, 1H), 4.78 – 4.84 (m, 1H), 4.61 – 4.63 (m, 1H), 4.38 – 4.44 (m, 1H), 4.04 – 4.17 (m, 4H), 3.66 (s, 3H), 3.65 (s, 3H), 3.48 (sbr, 6H), 3.31 – 3.35 (m, 1H), 3.13 (dd, 1H, $^{3}J_{HH}$ = 5.0 Hz, $^{2}J_{HH}$ = 11.2 Hz), 2.80 – 2.83 (m, 1H), 2.66 – 2.72 (m, 1H). $^{19}$F NMR (376 MHz, DMSO-d6): -60.73 (s, 3F). $^{13}$C NMR (100 MHz, DMSO-d6): 171.70, 157.97, 155.73, 154.24, 151.99, 149.67, 144.72, 143.72, 143.66, 143.09, 140.62, 140.60, 137.68, 135.52, 135.24, 130.10, 129.72, 127.76, 127.67, 127.55, 126.95, 126.93 (q, $^{2}J_{CF}$ = 31.4 Hz), 125.29, 125.19, 124.76, 124.72, 124.42 (q, $^{1}J_{CF}$ = 271.7 Hz), 120.06, 119.68, 113.06, 90.01, 85.50, 80.86, 72.69, 65.61, 62.76, 55.60, 54.91, 54.83, 50.34, 46.44, 37.84. ESI-MS ($ES^{+}$): m/z calcd for $C_{58}H_{54}F_{3}N_{7}O_{8}$ 1033.4, found 1034.4 ($MH^{+}$), 1056.4 ($M + Na^{+}$).

**General Procedure for the Preparation of Succinate Puromycin Derivatives 4a-j**

In a 10 ml round bottom flask under argon, anhydrous pyridine and succinic anhydride (3.0 eq) were added to the 5'-protected puromycin derivatives **3a-j** (1 eq). 4-(dimethylamino)-pyridine (DMAP, 0.5 eq) was added and the solution was stirred overnight at room temperature. Methanol (3 ml) was added and the solution was stirred at room temperature for 1 hour to quench the reaction. After evaporation in vacuo, the residue was purified by preparative TLC plate to produce the corresponding 2'-succinate puromycin derivatives **4a-j**.

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-3,4-dichlorophenylalanyl]-5'-O-(p,p'dimethoxytrityl)-$N^{6}$,$N^{6}$-dimethyladenosine-2'-O-succinate* **4a**. Isolated in 55% yield (152 mg) by preparative TLC plate ($CH_2Cl_2$/MeOH, 90:10), starting from 5'-protected puromycin derivative **3a** (250 mg, 0.241 mmol), succinic anhydride (73 mg, 0.723 mmol), DMAP (15 mg, 0.120 mmol), anhydrous pyridine (2.5 ml). $^{1}$H NMR (400 MHz, DMSO-d6): 8.58 (d, 1H, $^{3}J_{HH}$ = 4.2 Hz), 8.37 (d, 1H, $^{3}J_{HH}$ = 8.4 Hz), 8.31 (s, 1H), 8.23 (s, 1H), 7.87 (d, 2H, $^{3}J_{HH}$ = 7.5 Hz), 7.59 – 7.61 (m, 2H), 7.51 (d, 1H, $^{3}J_{HH}$ = 8.2 Hz), 7.16 – 7.42 (m, 15H), 6.82 – 6.78 (m, 4H), 6.20 (d, 1H, $^{3}J_{HH}$ = 2.9 Hz), 5.87 (m, 1H), 5.11 (m, 1H), 4.19 – 4.27 (m, 6H), 3.67 (s, 6H), 3.56 (sbr, 6H), 3.14 – 3.26 (m, 2H), 2.63 – 2.76 (m, 6H). $^{13}$C NMR (100 MHz, DMSO-d6): 172.59, 171.50, 171.25, 157.99, 155.76, 154.27, 152.11, 149.74, 149.60, 144.68, 143.85, 143.49, 140.63, 139.30, 138.52, 136.12, 135.33, 135.17, 131.18, 130.57, 130.14, 129.66, 129.02, 128.90, 127.74,

127.61, 127.00, 126.60, 125.19, 123.90, 120.08, 119.61, 113.04, 87.11, 85.44, 80.65, 73.70, 65.66, 62.77, 55.69, 54.87, 51.37, 49.62, 46.47, 35.76, 28.55. ESI-MS ($ES^-$): m/z calcd for $C_{61}H_{57}Cl_2N_7O_{11}$ 1135.0, found 1134.4 ($M\text{-}H^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-2,4-dichlorophenylalanyl]-5'-O-(p,p'dimethoxytrityl)-$N^6$,$N^6$-dimethyladenosine-2'-O-succinate* **4b**. Isolated in 66% yield (138 mg) by preparative TLC plate ($CH_2Cl_2$/MeOH, 90:10), starting from 5'-protected puromycin derivative **3b** (190 mg, 0.183 mmol), succinic anhydride (55 mg, 0.549 mmol), DMAP (11 mg, 0.091 mmol), anhydrous pyridine (2 ml). $^1$H NMR (500 MHz, DMSO-d6): 8.59 (d, 1H, $^3J_{HH}$ = 4.1 Hz), 8.32 (s, 1H), 8.30 (s, 1H), 8.23 (s, 1H), 7.88 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.15 – 7.80 (m, 19H), 6.78 – 6.82 (m, 4H), 6.20 (d, 1H, $^3J_{HH}$ = 3.2 Hz), 5.88 (m, 1H), 5.08 (m, 1H), 4.34 – 4.39 (m, 1H), 4.11 – 4.25 (m, 4H), 3.75 (s, 6H), 3.43 (sbr, 6H), 3.14 (m, 1H), 3.04 (m, 1H), 2.47 – 2.74 (m, 6H). $^{13}$C NMR (125 MHz, DMSO-d6): 172.56, 171.11, 170.98, 158.02, 157.98, 155.64, 154.29, 152.09, 149.80, 149.56, 144.68, 143.87, 143.53, 140.65, 138.41, 136.14, 135.41, 135.25, 134.39, 134.33, 132.53, 131.91, 129.69, 129.61, 128.51, 127.71, 127.65, 127.61, 127.59, 126.99, 126.85, 126.60, 125.23, 125.18, 123.90, 120.07, 119.63, 113.04, 86.84, 85.44, 80.56, 73.54, 65.72, 62.84, 54.91, 53.66, 51.35, 49.56, 46.50, 34.61, 28.54. ESI-MS ($ES^-$): m/z calcd for $C_{61}H_{57}Cl_2N_7O_{11}$ 1135.0, found 1134.4 ($M\text{-}H^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-2-nitrophenylalanyl]-5'-O-(p,p'dimethoxytrityl)-$N^6$,$N^6$-dimethyladenosine-2'-O-succinate* **4c**. Isolated in 79% yield (208 mg) by preparative TLC plate ($CH_2Cl_2$/MeOH, 90:10), starting from 5'-protected puromycin derivative **3c** (240 mg, 0.237 mmol), succinic anhydride (72 mg, 0.711 mmol), DMAP (15 mg, 0.118 mmol), anhydrous pyridine (2.4 ml). $^1$H NMR (400 MHz, DMSO-d6): 8.59 (sbr, 1H), 8.34 (d, 1H, $^3J_{HH}$ = 8.2 Hz), 8.28 (s, 1H), 8.23 (s, 1H), 7.96 – 7.98 (m, 1H), 7.89 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.63 – 7.66 (m, 2H), 7.08 – 7.53 (m, 17H), 6.78 – 6.82 (m, 4H), 6.19 (d, 1H, $^3J_{HH}$ = 3.0 Hz), 5.85 – 5.88 (m, 1H), 5.02 – 5.07 (m, 1H), 4.43 – 4.49 (m, 1H), 4.07 – 4.14 (m, 4H), 3.74 (s, 6H), 3.36 (sbr, 6H), 3.05 – 3.24 (m, 2H), 2.45 – 2.77 (m, 6H). $^{13}$C NMR (100 MHz, DMSO-d6): 173.56, 171.25, 171.05, 157.98, 157.95, 155.62, 154.25, 152.08, 149.73, 149.00, 144.70, 143.81, 143.54, 140.62, 138.35, 136.12, 135.37, 135.25, 132.98, 132.60, 132.35, 132.98, 132.60,

132.35, 129.68, 129.59, 127.72, 127.62, 127.03, 125.26, 124.64, 120.06, 119.60, 113.02, 86.76, 85.41, 80.67, 73.53, 65.73, 62.81, 54.88, 49.44, 46.45, 34.37, 28.74. ESI-MS ($ES^-$): m/z calcd for $C_{61}H_{58}N_8O_{13}$ 1111.1, found 1110.3 ($M\text{-}H^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-2-fluorophenylalanyl]-5'-O-(p,p'dimethoxytrityl)-$N^6$,$N^6$-dimethyladenosine-2'-O-succinate* **4d**. Isolated in 63% yield (210 mg) by preparative TLC plate ($CH_2Cl_2$/MeOH, 90:10), starting from 5'-protected puromycin derivative **3d** (300 mg, 0.304 mmol), succinic anhydride (90 mg, 0.912 mmol), DMAP (20 mg, 0.152 mmol), anhydrous pyridine (3 ml). $^1$H NMR (400 MHz, DMSO-d6): 8.58 (d, 1H, $^3J_{HH}$ = 3.9 Hz), 8.33 (d, 1H, $^3J_{HH}$ = 8.4 Hz), 8.29 (s, 1H), 8.22 (s, 1H), 7.88 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.78 – 7.82 (m, 1H), 7.63 – 7.69 (m, 3H), 7.18 – 7.42 (m, 14H), 7.08 – 7.13 (m, 1H), 6.94 – 6.98 (m, 1H), 6.77 – 6.82 (m, 4H), 6.20 (d, 1H, $^3J_{HH}$ = 3.0 Hz), 5.86 – 5.88 (m, 1H), 5.04 – 5.09 (m, 1H), 4.33 – 4.38 (m, 1H), 4.08 – 4.17 (m, 4H), 3.73 (s, 6H), 3.46 (sbr, 6H), 2.79 – 2.94 (m, 2H), 2.56 – 2.69 (m, 2H), 2.47 – 2.54 (m, 4H). $^{19}$F NMR (376 MHz, DMSO-d6): -117.49 (s, 1F). $^{13}$C NMR (100 MHz, DMSO-d6): 172.57, 171.26, 171.12, 160.67 (d, $^1J_{CF}$ = 245.1 Hz), 158.01, 157.98, 155.63, 154.27, 152.09, 149.77, 149.53, 144.70, 143.83, 143.57, 140.62, 138.41, 136.20, 135.40, 135.25, 131.46 (d, $^3J_{CF}$ = 3.8 Hz), 129.70, 129.60, 128.45 (d, $^3J_{CF}$ = 8.8 Hz), 127.71, 127.65, 127.61, 127.02, 126.59, 125.26, 124.33 (d, $^2J_{CF}$ = 15.1 Hz), 123.92, 120.05, 119.62, 114.93 (d, $^2J_{CF}$ = 22.6 Hz), 113.03, 86.85, 85.42, 80.62, 73.56, 65.71, 62.89, 54.89, 51.36, 49.55, 46.49, 30.83, 28.54. ESI-MS ($ES^-$): m/z calcd for $C_{61}H_{58}FN_7O_{11}$ 1084.1, found 1083.1 ($M\text{-}H^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-3,4-difluorophenylalanyl]-5'-O-(p,p'dimethoxytrityl)-$N^6$,$N^6$-dimethyladenosine-2'-O-succinate* **4e**. Isolated in 85% yield (235 mg) by preparative TLC plate ($CH_2Cl_2$/MeOH, 90:10), starting from 5'-protected puromycin derivative **3e** (250 mg, 0.249 mmol), succinic anhydride (75 mg, 0.747 mmol), DMAP (15 mg, 0.124 mmol), anhydrous pyridine (2.5 ml). $^1$H NMR (400 MHz, DMSO-d6): 8.58 (d, 1H, $^3J_{HH}$ = 4.1 Hz), 8.39 (d, 1H, $^3J_{HH}$ = 8.5 Hz), 8.31 (s, 1H), 8.23 (s, 1H), 7.88 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.77 – 7.81 (m, 1H), 7.60 – 7.63 (m, 2H), 7.08 – 7.42 (m, 16H), 6.78 – 6.81 (m, 4H), 6.21 (d, 1H, $^3J_{HH}$ = 2.6 Hz), 5.85 – 5.88 (m, 1H), 5.09 – 5.15 (m, 1H), 4.13 – 4.29 (m, 5H), 3.74 (s, 6H), 3.49 (sbr, 6H), 3.13 – 3.24 (m, 2H), 2.58

– 2.79 (m, 6H). $^{19}$F NMR (376 MHz, DMSO-d6): -139.40 (d, 1F, $^{3}J_{FF}$ = 22.6 Hz), -142.13 (d, 1F, $^{3}J_{FF}$ = 22.5 Hz). $^{13}$C NMR (100 MHz, DMSO-d6): 172.62, 171.64, 171.36, 157.98, 155.74, 154.26, 152.11, 150.19 (m), 149.60, 147.32 (m), 144.69, 143.85, 143.51, 140.63, 138.48, 136.12, 135.85 (m), 135.31, 135.17, 129.69, 129.63, 127.73, 127.60, 126.98, 126.94, 126.58, 125.96 (m), 125.21, 123.90, 120.07, 119.63, 118.11 (d, $^{2}J_{CF}$ = 16.9 Hz), 116.89 (d, $^{2}J_{CF}$ = 16.6 Hz), 113.03, 87.16, 85.43, 80.66, 73.73, 65.65, 62.68, 55.98, 54.86, 51.35, 49.56, 46.46, 36.62, 28.72. ESI-MS ($ES^-$): m/z calcd for $C_{61}H_{57}F_2N_7O_{11}$ 1102.1, found 1101.2 ($M\text{-}H^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-2-trifluoromethylphenylalanyl]-5'-O-(p,p'dimethoxytrityl)-N$^6$,N$^6$-dimethyladenosine-2'-O-succinate* **4f**. Isolated in 80% yield (220 mg) by preparative TLC plate ($CH_2Cl_2$/MeOH, 90:10), starting from 5'-protected puromycin derivative **3f** (250 mg, 0.241 mmol), succinic anhydride (72 mg, 0.723 mmol), DMAP (15 mg, 0.120 mmol), anhydrous pyridine (2.4 ml). $^{1}$H NMR (400 MHz, DMSO-d6): 8.58 (d, 1H, $^{3}J_{HH}$ = 4.1 Hz), 8.35 (d, 1H, $^{3}J_{HH}$ = 8.4 Hz), 8.29 (s, 1H), 8.23 (s, 1H), 7.89 (d, 2H, $^{3}J_{HH}$ = 7.5 Hz), 7.78 – 7.82 (m, 1H), 7.60 – 7.75 (m, 3H), 7.02 – 7.55 (m, 16H), 6.84 – 6.89 (m, 4H), 6.20 (d, 1H, $^{3}J_{HH}$ = 2.8 Hz), 5.88 (dd, 1H, $^{3}J_{HH}$ = 2.9 Hz, $^{2}J_{HH}$ = 6.5 Hz), 5.07 – 5.12 (m, 1H), 4.33 – 4.39 (m, 1H), 4.01 – 4.19 (m, 4H), 3.74 (s, 6H), 3.48 (sbr, 6H), 2.95 – 3.21 (m, 2H), 2.45 – 2.69 (m, 6H). $^{19}$F NMR (376 MHz, DMSO-d6): -58.10 (s, 3F). $^{13}$C NMR (100 MHz, DMSO-d6): 173.61, 171.26, 171.14, 157.98, 157.94, 155.60, 154.25, 152.08, 149.71, 149.60, 144.71, 143.86, 143.53, 140.64, 138.39, 136.12, 136.01, 135.36, 135.25, 131.84, 131.72, 129.66, 129.56, 127.68, 127.61, 127.31, 126.56 (q, $^{2}J_{CF}$ = 35.2 Hz), 125.85 (m), 125.34, 125.25, 124.32 (q, $^{1}J_{CF}$ = 273.8 Hz), 123.90, 120.06, 119.62, 113.00, 86.90, 85.39, 80.55, 73.59, 65.76, 62.78, 54.91, 49.52, 46.46, 33.95, 28.81. ESI-MS ($ES^-$): m/z calcd for $C_{62}H_{58}F_3N_7O_{11}$ 1134.1, found 1133.2 ($M\text{-}H^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-pentafluorophenylalanyl]-5'-O-(p,p'dimethoxytrityl)-N$^6$,N$^6$-dimethyladenosine-2'-O-succinate* **4g**. Isolated in 62% yield (67 mg) by preparative TLC plate ($CH_2Cl_2$/MeOH, 90:10), starting from 5'-protected puromycin derivative **3g** (100 mg, 0.094 mmol), succinic anhydride (30 mg, 0.282 mmol), DMAP (6 mg, 0.047 mmol), anhydrous pyridine (1 ml). $^{1}$H NMR (400 MHz,

DMSO-d6): 8.67 (d, 1H, $^3J_{HH}$ = 3.9 Hz), 8.51 (d, 1H, $^3J_{HH}$ = 8.4 Hz), 8.38 (s, 1H), 8.31 (s, 1H), 7.99 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.86 – 7.92 (m, 2H), 7.73 – 7.77 (m, 2H), 7.20 – 7.54 (m, 16H), 6.28 (d, 1H, $^3J_{HH}$ = 3.5 Hz), 5.97 (dd, 1H, $^3J_{HH}$ = 3.5 Hz, $^3J_{HH}$ = 6.6 Hz), 5.16 – 5.18 (m, 1H), 4.48 – 4.50 (m, 1H), 4.21 – 4.32 (m, 4H), 3.80 (s, 6H), 3.56 (sbr, 6H), 3.03 – 3.13 (m, 2H), 2.54 – 2.71 (m, 6H). $^{19}$F NMR (376 MHz, DMSO-d6): -141.46 (dd, 2F, $^4J_{FF}$ = 7.5 Hz, $^3J_{FF}$ = 24.2 Hz), -157.36 (t, 1F, $^3J_{FF}$ = 21.6 Hz), -163.44 (dt, 2F, $^4J_{FF}$ = 7.2 Hz, 3JFF = 22.7 Hz). $^{13}$C NMR (100 MHz, DMSO-d6): 172.57, 171.17, 169.95, 158.00, 157.98, 155.65, 154.28, 152.07, 149.77, 149.58, 146.38 (m), 144.65, 143.74, 143.53, 140.64, 138.46, 138.40 (m), 136.13, 135.38, 135.27, 129.67, 129.57, 127.62, 126.95, 125.14, 123.90, 120.07, 119.61, 112.99, 112.74, 86.75, 85.46, 80.71, 73.49, 65.87, 62.98, 54.89, 53.22, 51.36, 49.70, 46.46, 28.48. ESI-MS ($ES^-$): m/z calcd for $C_{61}H_{54}F_5N_7O_{11}$ 1156.1, found 1155.1 ($M\text{-}H^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-4-methylphenylalanyl]-5'-O-(p,p'dimethoxytrityl)-N$^6$,N$^6$-dimethyladenosine-2'-O-succinate* **4h**. Isolated in 78% yield (191 mg) by preparative TLC plate ($CH_2Cl_2$/MeOH, 90:10), starting from 5'-protected puromycin derivative **3h** (220 mg, 0.224 mmol), succinic anhydride (68 mg, 0.672 mmol), DMAP (14 mg, 0.112 mmol), anhydrous pyridine (2.3 ml). $^1$H NMR (500 MHz, DMSO-d6): 8.59 (d, 1H, $^3J_{HH}$ = 4.1 Hz), 8.35 (d, 1H, $^3J_{HH}$ = 8.5 Hz), 8.29 (s, 1H), 8.23 (s, 1H), 7.87 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.76 – 7.81 (m, 1H), 7.63 – 7.65 (m, 2H), 7.17 – 7.41 (m, 15H), 7.03 (d, 2H, $^3J_{HH}$ = 7.8 Hz), 6.77 – 6.80 (m, 4H), 6.20 (d, 1H, $^3J_{HH}$ = 2.6 Hz), 5.85 – 5.87 (m, 1H), 5.08 – 5.13 (m, 1H), 4.23 – 4.28 (m, 1H), 4.11 – 4.18 (m, 4H), 3.67 (s, 6H), 3.42 (sbr, 6H), 3.14 – 3.23 (m, 2H), 2.58 – 2.78 (m, 6H), 2.23 (s, 3H). $^{13}$C NMR (125 MHz, DMSO-d6): 173.00, 172.38, 171.69, 158.39, 158.36, 156.10, 154.68, 152.47, 150.12, 149.98, 145.08, 144.26, 143.98, 141.00, 138.79, 137.80, 136.48, 135.79, 135.61, 135.48, 135.27, 130.08, 129.99, 129.43, 128.96, 128.09, 128.03, 127.96, 127.93, 127.39, 126.96, 125.71, 125.67, 124.26, 120.40, 120.04, 113.43, 87.46, 85.83, 81.09, 74.08, 66.02, 63.13, 56.66, 55.27, 51.72, 49.92, 46.91, 37.58, 29.17, 21.02. ESI-MS ($ES^-$): m/z calcd for $C_{62}H_{61}N_7O_{11}$ 1080.2, found 1079.2 ($M\text{-}H^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-4-(N-terbutylmethoxycarbonyl)-phenylalanyl]-5'-O-(p,p'dimethoxytrityl)-$N^6$,$N^6$-dimethyladenosine-2'-O-succinate* **4i**. Isolated in 74% yield (212 mg) by preparative TLC plate ($CH_2Cl_2$/MeOH, 90:10), starting from 5'-protected puromycin derivative **3i** (260 mg, 0.240 mmol), succinic anhydride (73 mg, 0.720 mmol), DMAP (15 mg, 0.120 mmol), anhydrous pyridine (2.4 ml). $^1$H NMR (500 MHz, DMSO-d6): 9.24 (sbr, 1H), 8.58 (d, 1H, $^3J_{HH}$ = 4.1 Hz), 8.36 (d, 1H, $^3J_{HH}$ = 8.3 Hz), 8.28 (s, 1H), 8.23 (s, 1H), 7.87 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.77 – 7.81 (m, 1H), 7.61 – 7.63 (m, 2H), 7.17 – 7.41 (m, 17H), 6.78 – 6.80 (m, 4H), 6.19 (d, 1H, $^3J_{HH}$ = 2.4 Hz), 5.85 – 5.87 (m, 1H), 5.10 – 5.15 (m, 1H), 4.12 – 4.25 (m, 5H), 3.71 (s, 6H), 3.44 (sbr, 6H), 3.13 – 3.23 (m, 2H), 2.45 – 2.74 (m, 6H), 1.47 (s, 9H). $^{13}$C NMR (125 MHz, DMSO-d6): 173.02, 172.47, 171.78, 158.37, 158.35, 156.10, 154.67, 153.15, 152.48, 150.10, 149.99, 145.06, 144.28, 143.97, 140.99, 138.76, 138.15, 136.49, 135.77, 135.59, 131.94, 130.07, 129.99, 129.73, 129.29, 128.10, 128.02, 127.94, 127.89, 127.65, 127.43, 127.40, 126.95, 125.70, 124.27, 121.75, 120.39, 120.04, 118.21, 113.44, 87.52, 85.84, 81.07, 79.19, 74.14, 66.03, 62.99, 56.81, 55.27, 51.71, 49.90, 46.89, 37.35, 29.23, 28.51. ESI-MS ($ES^-$): m/z calcd for $C_{66}H_{68}N_8O_{13}$ 1181.3, found 1180.3 ($M\text{-}H^+$).

*3'-Amino-3'-deoxy-3'-[N-(9-fluorenylmethoxycarbonyl)-L-4-trifluoromethylphenylalanyl]-5'-O-(p,p'dimethoxytrityl)-$N^6$,$N^6$-dimethyladenosine-2'-O-succinate* **4j**. Isolated in 71% yield (133 mg) by preparative TLC plate ($CH_2Cl_2$/MeOH, 90:10), starting from 5'-protected puromycin derivative **3j** (170 mg, 0.164 mmol), succinic anhydride (50 mg, 0.492 mmol), DMAP (10 mg, 0.082 mmol), anhydrous pyridine (1.7 ml). $^1$H NMR (400 MHz, DMSO-d6): 8.58 (d, 1H, $^3J_{HH}$ = 3.9 Hz), 8.46 (d, 1H, $^3J_{HH}$ = 8.4 Hz), 8.30 (s, 1H), 8.21 (s, 1H), 7.93 – 7.95 (m, 1H), 7.87 (d, 2H, $^3J_{HH}$ = 7.5 Hz), 7.48 – 7.63 (m, 5H), 7.08 – 7.41 (m, 14H), 6.78 – 6.81 (m, 4H), 6.20 (d, 1H, $^3J_{HH}$ = 2.4 Hz), 5.86 – 5.88 (m, 1H), 5.12 – 5.18 (m, 1H), 4.30 – 4.35 (m, 1H), 4.05 – 4.20 (m, 4H), 3.66 (s, 6H), 3.48 (sbr, 6H), 3.13 – 3.25 (m, 2H), 2.58 – 2.90 (m, 6H). $^{19}$F NMR (376 MHz, DMSO-d6): -60.75 (s, 3F). $^{13}$C NMR (100 MHz, DMSO-d6): 172.64, 171.68, 171.41, 157.96, 155.75, 154.26, 152.10, 149.68, 149.60, 144.67, 143.83, 143.53, 143.02, 140.61, 138.46, 136.12, 135.33, 135.17, 130.02, 129.68, 129.62, 127.73, 127.61, 127.16, 126. 82 (q, $^2J_{CF}$ = 31.2 Hz), 125.27, 124.84, 124.37 (q, $^1J_{CF}$ = 271.7 Hz), 120.04, 119.64, 113.04,

87.19, 85.43, 80.63, 73.75, 65.64, 62.63, 55.85, 54.84, 51.34, 49.56, 46.44, 37.29, 28.79. ESI-MS ($ES^-$): m/z calcd for $C_{62}H_{58}F_3N_7O_{11}$ 1134.1, found 1133.2 ($M\text{-}H^+$).